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Vitamin D, Calcium, and Dairy Consumption and Risk of Early Menopause

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VITAMIN D, CALCIUM, AND DAIRY CONSUMPTION AND RISK OF EARLY MENOPAUSE

A Dissertation Presented

by

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DEDICATION

This dissertation is dedicated to my mother and sister, whose unconditional love and support has allowed me to pursue my dreams.

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I would like to express my profound gratitude for the efforts of my advisor, Dr. Elizabeth Bertone-Johnson, throughout my time as a graduate student. She is a phenomenal teacher, researcher, and mentor, and I am so fortunate to have worked with her over the past four years. I am forever indebted to her for her patience, guidance, and commitment to my success as researcher. Without her, this dissertation would not have been possible.

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ABSTRACT

VITAMIN D, CALCIUM, AND DAIRY CONSUMPTION AND RISK OF EARLY MENOPAUSE

MAY 2018

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Early menopause, defined as the cessation of ovarian function before the age of 45, affects roughly 10% of women in Western populations. Current research suggests that women who experience early menopause are at increased risk for cardiovascular disease and other adverse health outcomes. Early menopause may also have substantial financial and psychological consequences for family planning, particularly as women increasingly delay childbearing into the later reproductive years. Emerging research suggests that modifiable lifestyle factors such as diet may play an important role in ovarian aging. According to our review of the current biologic and epidemiologic literature in Chapter 1, vitamin D, calcium, and dairy consumption may be related to the physiologic processes involved in ovarian aging. However, no prior epidemiologic studies have evaluated these exposures with regard to risk of early menopause. Thus, the aim of this dissertation was to evaluate these associations in the prospective Nurses' Health Study II (NHS2) (n=112,429).

In Chapter 2, we evaluated how intakes of vitamin D and calcium are associated with risk of early menopause. Results of this study suggest that high versus low intakes of vitamin D and calcium from food sources, particularly dairy foods, are associated with 17% and 13% lower risk of early menopause, respectively.

In Chapter 3, we evaluated how total and free plasma 25-hydroxyvitamin D (25(OH)D) and vitamin D binding protein levels (VDBP) are associated with risk of early menopause. According to our findings, total and free 25(OH)D levels are not associated with risk of early menopause, and VDBP may be positively associated with risk.

In Chapter 4, we evaluated how intakes of total, low-fat, high-fat, and individual dairy foods are associated with risk of early menopause. Findings indicate that high versus low intake of low-fat dairy foods is associated with 23% lower risk of early menopause. In particular, intakes of skim milk and yogurt intake were associated with lower risk of early menopause.

In conclusion, vitamin D and calcium are not importantly related to early menopause risk. Intake of low-fat dairy foods is associated with lower risk of early menopause, but findings should be replicated in future studies.

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CHAPTER 1

DAIRY CONSUMPTION AND OVARIAN AGING AND REPRODUCTIVE LIFESPAN: A NARRATIVE REVIEW

1.1 Abstract

Accelerated ovarian aging is characterized by earlier onset of menopause and is associated with increased risk of cardiovascular disease, early cognitive decline, and other conditions. In addition, accelerated ovarian aging may interfere with family planning, particularly as women choose to delay childbearing until the later reproductive years. Recent evidence suggests that the rate of ovarian aging is modifiable, particularly with regard to dietary factors. Dairy is comprised of several nutritive and non-nutritive components that may be functionally related to ovarian aging. This aim of this review was to summarize potential biologic mechanisms through which dairy may influence ovarian function, systematically evaluate the existing epidemiologic evidence, and identify potential questions to be addressed in future studies. Evidence from in vitro and animal studies suggest that dairy food intake may be related to ovarian aging through pathways related to vitamin D, inflammation, and sex hormones. However, at this time, epidemiologic evidence is limited; for example, no studies have evaluated dairy intake and early menopause, and only one prospective study has evaluated age at menopause with regard to low and high-fat dairy. Results from this study suggest that intake of low-fat dairy foods, but not high-fat dairy may extend reproductive lifespan, but important questions remain. To address current gaps in our understanding of this important topic, future prospective studies should examine specific dairy foods, compare associations of

dairy intake at different ages, and directly evaluate how dairy relates biologically to ovarian function. Findings from these studies will provide a clearer picture of how dairy intake may be related to ovarian aging and identify the mechanisms through which dairy may influence reproductive lifespan.

1.2 Introduction

Emerging evidence suggests that the duration of a woman's reproductive lifespan is related to risk of chronic diseases. Specifically, a shorter reproductive lifespan, usually marked by the early cessation of ovarian function, is associated with increased risk of cardiovascular disease, early cognitive decline, and osteoporosis, among other conditions. (1–5) Findings from population-based studies indicate that genetic factors do not explain the majority of variation in menopausal timing, (6,7) and that modifiable lifestyle factors, including diet, may play a role in maintaining normal ovarian function during the third and fourth decades of a woman's life. (8–10) Recent evidence suggests that intake of dairy foods may be related to several reproductive outcomes; (11–13) however, to our knowledge, no prior reviews have evaluated the existing evidence regarding how dairy consumption is associated with ovarian aging and reproductive lifespan. This review briefly discusses recent advances in our understanding of the physiology of ovarian aging, summarizes potential biologic mechanisms through which dairy may influence ovarian function, systematically evaluates the existing epidemiologic evidence, and proposes potential avenues of future research to address gaps in our current understanding of this important topic.

1.3 Current Status of Knowledge

1.3.1 Physiology of ovarian aging

Ovarian aging refers to the process through which the number and quality of follicle-surrounded oocytes present in the ovarian cortex declines as a woman ages. (6,14) According to Broekmans et al, at about the fourth month of fetal development, the

female ovaries contain roughly 6-7 million oocytes surrounded by granulosa cells, which constitute the primordial follicle pool. (6) During the second half of fetal development, the majority of primordial follicles undergo apoptosis; at birth, only 1-2 million primordial follicles remain in the ovarian cortex. (15) This age-related decline in primordial follicles continues through childhood, adolescence, and the reproductive years until menopause occurs. At menarche, approximately 300,000 follicles remain, and at the onset of menopause, this number is reduced to ~1,000. (16–18)

During the reproductive years, >99% of primordial follicles are non-growing. (6) At any given time, ~20-150 follicles are in the early stages of growth, during which the size of the oocytes increase and the granulosa cells of the follicles proliferate. (19,20) The small preantral follicles continue to enlarge and the granulosa cells surrounding the oocyte develop follicle-stimulating hormone (FSH) receptors until the follicle reaches the large antral stage. (19) The development of the antral cavity indicates transition to the antral follicle stage, at which point the follicle becomes responsive to FSH. (19) A single dominant follicle is then selected and the oocyte is released from the follicle as ovulation occurs. After ovulation, the follicle then develops into the corpus luteum. (6) Ovulation is followed by fertilization and implantation, or in the absence of fertilization, regression of the corpus luteum and endometrial shedding (i.e., menses). (6) As the ovary ages and follicle count declines, the quality of oocytes and the follicles that surround the oocytes also decline, perhaps increasing the rate of meiotic nondisjunction. (14,20–24)

Ovarian aging and function are commonly assessed using biomarker measures such as anti-Müllerian hormone (AMH), FSH, and antral follicle count (AFC).

Assessment of ovarian age via biomarkers has important implications not only for clinical understanding of reproductive status and potential, but also for research purposes.

1.3.1.1 Anti-Müllerian Hormone

In recent years, anti-Müllerian hormone has been identified as a marker of ovarian age and predictor of timing of menopause. AMH is a glycoprotein secreted exclusively by the granulosa cells of primary, secondary and small antral follicles of the ovary. (25) AMH secretion begins as primordial follicles differentiate to the primary stage and ceases once the follicles reach the mid-antral stage, just before cyclic recruitment. (26–28) AMH has been well established as a marker of reproductive decline due to its high correlation with antral follicle count, which is an indicator of the number of primordial follicles remaining in the ovarian cortex. (28–34) As the primordial follicle pool diminishes, serum levels of AMH decline until they become undetectable at the onset of menopause. (35–38)

In addition to being a marker of ovarian reserve, recent studies suggest that AMH may also influence the rate of ovarian aging by regulating early follicle growth. This hypothesis is supported by findings of Durlinger et al, who demonstrated that AMH inhibits primordial follicle growth in mice. (26) A subsequent study by the same group found that AMH knock-out mice experienced accelerated follicle recruitment and premature depletion of ovarian follicles as compared to their wild-type counterparts, and that AMH diminishes FSH-stimulated preantral follicle growth in vitro. (27)

1.3.1.2 Follicle-Stimulating Hormone

Assessment of FSH levels may also provide information on ovarian age and reproductive potential. Throughout the reproductive years, FSH is responsible for cyclic recruitment of antral follicles, follicle development to the pre-ovulatory phase, and sensitization of the follicle to luteinizing hormone. (39,40) While FSH levels are variable across the menstrual cycle, ovarian aging during the later reproductive years is characterized by higher basal levels of FSH during the early follicular phase. (41–43) This is primarily due to the decline in inhibin B, a dimeric polypeptide secreted by granulosa cells of the antral follicles. (44–46) Ordinarily, inhibin B suppresses FSH production by the pituitary gland; however, as the ovarian follicle pool diminishes with age, inhibin B correspondingly declines, resulting in higher levels of FSH. (46) The rise in FSH results in a shortening of the follicular phase and therefore greater ovulatory frequency. (47–49)

Emerging data suggest that FSH is not only reflective of the age-related decline in follicle numbers during the late reproductive years, but may also exert direct effects on the acceleration of follicle loss in the years leading up to menopause. McTavish et al recently demonstrated that transgenic (tg)-FSH mice <22 weeks of age expressing increasing levels of pituitary-independent human FSH experienced higher rates of ovulation and larger litter sizes as compared to their wild-type (WT) counterparts. (39) However, for mice ≥22 weeks of age, litter sizes were smaller and earlier loss of fertility occurred in tg-FSH mice as compared to WT mice. Two important points may be gleaned from these data: 1) these findings are consistent with the hypothesis that FSH increases rates of ovulation by increasing dominant follicle development, leading to decline in

fertility, and 2) that FSH may directly accelerate follicle loss, rather than simply reflect the diminishing follicle pool. While these findings have not yet been confirmed in humans, they are supported by the paradoxical observation that women with fragile X syndrome, which is associated with higher levels of FSH, commonly experience higher rates of multiple ovulation (dizygotic twinning), yet earlier onset of menopause. (50–52)

One of the complicating factors in using FSH as a marker of ovarian aging is that basal FSH levels rise during the very late reproductive years, only after a significantly proportion of the follicle pool is lost. (6) During the normal menstrual cycle, FSH is secreted by the pituitary gland, stimulating follicle growth and production of estradiol by granulosa cells. Rising estradiol and progesterone work in a negative feedback loop to slow the release of FSH. Correspondingly, as estradiol levels decline, FSH levels increase. FSH levels are therefore not only influenced by the size of the follicle pool, but also by fluctuations in estradiol, which presents methodologic challenges for epidemiologic studies using FSH as a marker of ovarian aging.

1.3.1.3 Antral Follicle Count

While AMH and FSH levels serve as proxies for ovarian reserve, AFC provides a direct measurement of the size of the oocyte pool. As described earlier, the size of the primordial follicle pool declines steadily with age and is indicative of ovarian aging. Although the primordial follicles themselves are too small to identify in a clinical setting using diagnostic imaging, the primordial follicle pool size is highly correlated with AFC throughout the reproductive years. (6,53) Antral follicles are large enough (2-10 mm) to be detected by transvaginal sonography imaging, and are sensitive to FSH. (54) As the

primordial follicle pool size decreases with age, AFC similarly declines, and is thus a reliable measure of ovarian reserve. (53)

Given that AMH is secreted exclusively by the granulosa cells of the preantral follicles, AFC is highly correlated with AMH levels at all ages. As compared to FSH, AFC and AMH levels are better predictors of ovarian aging throughout the reproductive years because they are able to assess ovarian reserve before a significant decline in follicle count has occurred.

1.3.2 Consequences of accelerated ovarian aging

Accelerated ovarian aging is characterized by an increased rate of follicle loss and earlier age at menopause. The mean age of menopause in Western populations is 51, although the range is fairly wide (~40-60 years). (25) About 10% of women experience early natural menopause (i.e., menopause before age 45). (6) Premature ovarian failure (POF) affects 1% of women, and is defined as menopause before the age of 40. (6) The consequences of accelerated ovarian aging and thus, early menopause or POF are substantial. Epidemiologic evidence suggests that women who experience early menopause are at increased risk of cardiovascular disease, early cognitive decline, and osteoporosis, among other conditions. (1–5) Because women experience an ebb in fertility during the 10 years leading up to menopause, early menopause may interfere with family planning, which may have serious financial and psychological implications. (1,6)

Recent studies suggest that ovarian aging and menopausal timing are modifiable – specifically, smoking (55–59), diet (8–10,60,61), and BMI (57,62) have been associated

with the age at which menopause occurs. With regard to diet, dairy food intake is of particular interest, as it has been associated with other reproductive health conditions in women, such as endometriosis and premenstrual syndrome. (13,63) The aim of this review is to evaluate the potential physiologic mechanisms and epidemiology of dairy foods and ovarian aging, specifically.

1.3.3 Dairy foods and ovarian aging

According to the USDA, the dairy food group includes as all fluid milk products and foods made from milk. (64) Dairy foods are a rich source of micronutrients including vitamin D, vitamin B_{12}, calcium, phosphorus, potassium, and magnesium. Macronutrient constituents of dairy foods include milkfat, lactose, and whey and casein protein. (65) In addition to macro and micronutrients, dairy also contains non-nutritive components, including estrogens, progesterone, testosterone, and androstenedione. (66,67)

Currently, the USDA recommends that women ages 9 years and older consume at least three servings of dairy foods per day (e.g., 1 cup of milk or yogurt, or 1.5 ounces of natural cheese). (68) Findings from the National Health and Nutrition Examination Survey indicate that among women ages 14-18, mean intake of dairy foods is currently 1.6 servings/day. (69) Intake declines with age, as women in the 19-30 age group consume 1.5 servings/day, and women 31-50 years old consume 1.4 servings/day, on average.

Findings of animal and in vitro studies suggest a number of mechanisms through which these dairy constituents may directly or indirectly influence ovarian aging, which are summarized below.

1.3.3.1 Vitamin D

Fortified milk is an important source of dietary vitamin D; for example, one 8 oz. serving provides ~100 IU of vitamin D. (65,70) In fact, fortified dairy products contribute more vitamin D to the diets of U.S. adults than any of the other food groups alone. Recent systematic reviews have concluded that vitamin D plays a role in conditions related to fertility, including polycystic ovary syndrome (PCOS) and in vitro fertilization outcomes; however, potential mechanisms explaining such associations remain poorly understood. (71,72)

Vitamin D metabolism has been described in detail previously. (73) Briefly, vitamin D may be obtained in one of two ways. The majority of vitamin D is produced during periods of solar UVB radiation exposure by cutaneous conversion of 7-dehydrocholesterol to provitamin D_3, which is then isomerized to colecalciferol (vitamin D_3). A smaller proportion of vitamin D may be obtained through supplements and dietary intake of foods that naturally contain or are fortified with vitamin D, as either vitamin D_2 (ergocalciferol) or vitamin D_3.

Upon ingestion or solar UVB radiation exposure, colecalciferol and ergocalciferol are converted to 25-hydroxyvitamin D (25(OH)D) by a-hydroxylase, primarily in the liver. 25(OH)D is the primary circulating form of vitamin D, and is later hydroxylated in the kidney and other tissues to the active metabolite of the vitamin, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$). Circulating $1,25(OH)_2D$ then binds to vitamin D receptors (VDR) present in many tissues of the body, including the ovary. (74)

Findings from animal and in vitro studies suggest that vitamin D may play a role in ovarian aging, through associations with AMH, FSH and estradiol levels. Malloy et al

identified a vitamin D response element (VDRE) in the promoter region of the AMH gene, providing evidence that vitamin D has a direct effect on the mRNA expression of AMH. (75) Findings from an in vitro study by Krishnan et al indicate that vitamin D upregulates AMH mRNA in prostate cells, consistent with the hypothesis that vitamin D plays a regulatory role in the expression of AMH. (76) In line with these findings, Wojtusik et al observed that vitamin D modulates AMH mRNA expression in a dose-dependent fashion in the granulosa cells of hen. (77) Specifically, a 100nM dose of vitamin D resulted in decreased expression of AMH mRNA among 3-5 mm and 6-8 mm follicles. Furthermore, FSH receptor expression and granulosa cells proliferation was higher among samples receiving 100nM of vitamin D as compared to the control group.

The findings of these studies provide compelling evidence of a potential role of vitamin D in the expression of AMH and folliculogenesis. It is possible that elevated 25(OH)D levels contribute to higher circulating $1,25(OH)_2D$, which binds to AMH gene VDRE in the granulosa cells of primary, secondary, and small antral follicles of the ovary. This may in turn up-regulate AMH secretion by the granulosa cells and slow follicle growth and recruitment, and work to preserve the number of follicles present in ovarian cortex, thus prolonging the reproductive lifespan. These findings also raise important questions. Given that these were in vitro studies, it is still unclear whether the doses tested are physiologically relevant for humans. Moreover, it remains unclear whether vitamin D-induced changes in AMH expression and secretion may be sufficient to interfere with follicle recruitment in humans or alter menopausal timing.

Vitamin D from dairy foods may also be related to ovarian aging through indirect effects on circulating FSH levels via estradiol. As described earlier, elevated FSH levels

are indicative of ovarian aging, albeit only during the late reproductive years.(78) FSH levels are variable across the menstrual cycle, and are influenced by estrogen levels via negative feedback. (6) Estrogens including estradiol are produced by aromatization of androgen precursors by aromatase (CYP19). (79) and recent in vitro studies have found that aromatase activity is altered by $1,25(OH)_2D$ levels in prostate and placental cells and osteoblasts. (80,81) Correspondingly, findings of Kinuta et al indicate that aromatase activity is suppressed in VDR null mutant mice as compared to WT counterparts, suggesting that aromatase activity is sensitive to vitamin D deficiency. (82) It is therefore possible that estrogen synthesis may be functionally related to 25(OH)D status through effects on conversion of androgen precursors to estrogens by aromatase. (79) In line with this hypothesized mechanism, findings from one cross-sectional study of Norwegian women suggest that estradiol levels vary significantly according to season, with highest levels recorded in June. (83)

Because FSH levels are inversely related to estrogen levels, 25(OH)D levels may influence levels of estradiol, which have an inhibitory effect on levels of FSH. In light of the evidence suggesting that increasing levels of FSH may accelerate ovarian aging, vitamin D may work to increase levels of estradiol, which reduces basal FSH levels and therefore slows the decline in the follicle pool size during the later reproductive years.

1.3.3.2 Sex steroids in dairy foods

Bovine milk contains steroid hormones and growth factors that may also be related to ovarian aging including estrogens, progesterone, and androgen precursors. Laboratory evidence suggests that milk products contain varying concentrations of

conjugated and unconjugated estrogen metabolites. For example, in one study, levels of unconjugated estrogen metabolites in skim milk were found to be relatively modest (7.21 pg/mL), but were higher with increasing milkfat content (51.64 pg/mL for whole milk and 273.72 pg/mL for buttermilk). (66) Dairy milk also contains appreciable amounts of progesterone; for example, whole milk contains 10 μg of progesterone per liter. (67) Because unconjugated estrogen metabolites and progesterone are lipophilic, concentrations are considerably higher in full-fat dairy products. (67) However, it is important to note that conjugated estrogen metabolites, including estrone sulfate, are hydrophilic and thus may be more bioavailable in low-fat dairy products. (66) Conjugated estrogen metabolites are considered to be more biologically active than their unconjugated counterparts due to circumvention of hepatic metabolism. (84) Importantly, these hormones and metabolites have been shown to remain biologically active after ingestion, and may induce changes in the hormonal profile. (85) This evidence is further supported by findings from an epidemiologic study in which consumption of dairy products was significantly positively associated with plasma levels of total and free estradiol. (86)

In addition to estrogen and progesterone, milk also contains androgens that may be implicated in ovarian aging, including testosterone and androstenedione. (87) Concentrations of these androgens vary, but have been estimated to fall between 0.02-0.15 μg/L and 0.1-3.5 μg/L, for testosterone and androstenedione, respectively. (87) Exogenous androgens have been shown to increase levels of circulating insulin-like growth factor 1 (IGF-1) in humans. (88) In fact, four studies have observed that dairy intake is associated with significantly higher levels of circulating IGF-1. (89–92) Age is

associated with a decrease in circulating IGF-1, (8,93) and studies in rats have observed that low IGF-1 is associated with disruption of luteinizing hormone, (93) the hormone primarily responsible for ovulation. It is possible that dairy consumption may therefore increase levels of IGF-1, allowing for the continuation of normal menstrual cycles during the later reproductive years.

Taken together, these studies provide provocative evidence that the non-nutritive components of dairy may alter hormone levels in premenopausal women. It is currently unclear, however, whether the fluctuations in steroid hormones associated with dairy consumption are sufficient to induce consequential alterations in ovarian function or the rate of ovarian aging.

1.3.3.3 Inflammation

Dairy may also play a role in ovarian aging through the modification of inflammatory pathways that influence levels of circulating cytokines. Chronic systemic inflammation is associated with upregulation of the nuclear factor-kappaB (NF-kB) pathway and increased levels of tumor necrosis factor-alpha (TNF-a), interleukin-6 (IL-6) and IL-6-stimulated production of C-reactive protein (CRP) in the liver. (94) A recent meta-analysis of genome-wide association studies identified a candidate gene (PRRC2A) implicated in both menopausal timing and the NF-kB inflammatory pathway, lending support for a potential role of inflammation in ovarian aging. (95) In addition, one study observed higher serum and follicular fluid levels of CRP among anovulatory women, as compared to ovulatory women, suggesting that the ovaries may be sensitive to such markers of inflammation. (96)

Emerging evidence suggests that dairy foods have anti-inflammatory properties. One recent randomized trial observed that among overweight and obese adults with metabolic syndrome, those fed an adequate-dairy diet (>3.5 servings/day) as compared to a low-dairy diet (<0.5 servings/day) experienced a significant reduction in levels of circulating IL-6 and TNF-a after 12 weeks. (97) Similarly, another randomized cross-over trial found that among overweight and obese individuals, consuming a dairy-supplemented versus a eucaloric soy-supplemented diet was associated with significantly lower levels of TNF-a and IL-6. (98) In light of this evidence, it is plausible that dairy may alter the systemic and ovarian inflammatory milieu, thereby influencing rate of ovarian aging.

Dairy may influence ovarian aging through inflammatory pathways through effects on adiposity, and through anti-inflammatory action of vitamin D. The association of adiposity and systemic inflammation is well-established, as adipocytes directly produce pro-inflammatory cytokines including TNF-a and IL-6, among others. (99) In particular, hypertrophy of visceral adipocytes results in the release of pro-inflammatory cytokines including IL-6 and TNF-a, suggesting that central adiposity may be more inflammatory than subcutaneous adiposity. (94) A recent meta-analysis of randomized controlled trials found dairy intake to be associated with a significant reduction in both overall fat mass and visceral adiposity. (100) The physiology underlying this association is currently unclear, though some have hypothesized specific mechanisms relating to milkfat, whey protein, and calcium. (65,101)

Whole milk contains roughly 34 g/L of fat, about 50% of which is saturated. (102) Specifically, the saturated fatty acids most abundant in milk include palmitic acid (16:0),

stearic acid (18:0), and myristic acid (14:0).(102) Roughly 25% of the fat content of milk is comprised of monounsaturated fatty acids including oleic acid (cis 9-18:1) and trans-palmitoleic acid (trans-9, 16:1). (102) Oleic acid is unique in that the double bond position may result in preferential oxidation, relative to other fatty acids, and has been shown to accumulate in subcutaneous rather than visceral fat stores. (103,104) Some evidence also suggests that trans-palmitoleic acid is associated with lower waist circumference and BMI. (105) Oleic and trans-palmitoleic acid in dairy may divert fat deposition to the subcutaneous rather than the visceral adipocytes, resulting in lower systemic levels of pro-inflammatory cytokines including CRP.

Milk also contains roughly 34 g/L of protein, which is comprised of 80% casein protein and 20% whey protein. (106) In particular, whey protein may be relevant to the adiposity-inflammation relation with ovarian aging due to anti-obesogenic properties. To that extent, whey protein contains peptides including lactokinins, which act as angiotensin converting enzyme (ACE)-inhibitors. (107) Ordinarily, angiotensin II hormone increases adipocyte lipogenesis through upregulation of fatty acid synthase expression (108); however, in vitro studies have found that in the presence of whey protein, ACE is inhibited, (107) leading to suppression of angiotensin II hormone, and thus decreased endogenous fat production. (101) These findings are further supported by studies that have found whey protein to be effective in reducing weight while retaining lean body mass. (109) According to this evidence, it is possible that whey protein in dairy may inhibit adipocyte lipogenesis, resulting in reduced adiposity and levels of the inflammatory cytokines implicated in ovarian aging.

Dairy is also an excellent source of calcium; for example, each 8 oz. serving of milk contains ~30% of the RDA for women ages 19 and older. (110) Findings from a number of epidemiologic studies suggest that intake of calcium, particularly from dairy, is inversely associated with adiposity. (111–113) Zemel et al. propose that the mechanism responsible for the anti-obesegenic effect of calcium relates to the role of intracellular calcium in adipocyte metabolism – specifically, the modulation of adipocyte triglyceride stores. (114) The authors note that dietary calcium intake suppresses calcitrophic hormones involved in adipocyte fat storage, such as parathyroid hormone. (114) Suppression of these hormones may result in lower overall adiposity and adiposity-induced inflammatory cytokines.

Collectively, the above evidence suggests a complex interplay of dairy components that may contribute to lower overall and central adiposity, thus protecting against chronic inflammation characterized by elevated levels of IL-6, TNF-a and IL-6-induced CRP. Some (31,34,57,62,115–121), but not all (122) studies have observed associations of BMI and adiposity and menopausal timing. In light of the evidence suggesting that the ovary is sensitive to these inflammatory cytokines, it is possible that dairy may protect against ovarian aging by altering the adiposity-mediated inflammatory pathway.

In addition to associations with AMH and FSH, vitamin D from milk products may also influence reproductive lifespan through the modulation of immune pathways. Vitamin D plays a multifunctional role in the immune pathways involved in inflammation; in particular, it has been shown to inhibit of T cell proliferation and monocyte activity. (123) One of the key roles of immune-helper T cells is to secrete

inflammatory cytokines including TNF-a. (123) Studies have shown that in the presence of vitamin D, T cell proliferation is suppressed, (124) which may in turn down-regulate secretion of TNF-a. Likewise, monocytes are responsible for production of IL-6, IL-1, IL-8, and TNF-a (123) and contain VDR, which mediate the biologic activity of $1,25(OH)_2D$. (73) Human monocyte activity has been shown to be inhibited in the presence of vitamin D in vitro, resulting in attenuated secretion of these inflammatory cytokines. (125)

These laboratory data are supported by epidemiologic evidence suggesting a direct link between circulating vitamin D and inflammatory factors including TNF-a and CRP. (126,127) For example, results from one cross-sectional study indicate that 25(OH)D levels are inversely associated with TNF-a concentrations. (126) A subsequent study by Ngo et al observed a similar inverse relationship of 25(OH)D and CRP levels. (127) Given the potency of vitamin D in fortified milk, it is possible that dairy may influence ovarian aging in part through immunomodulatory effects of vitamin D that reduce chronic systemic inflammation. In fact, in recent years, vitamin D has been consistently associated with a number of conditions related to immune dysfunction including multiple sclerosis, (128) rheumatoid arthritis, (129) and inflammatory bowel disease. (130)

1.4 Epidemiologic Evidence: Dairy Foods and Reproductive Lifespan

Despite strong evidence from laboratory and animal studies, relatively few epidemiologic studies have evaluated menopause timing and ovarian aging with respect to dairy consumption. A number of other studies have evaluated dairy and other

conditions potentially related to ovarian aging, though it is unclear whether the pathophysiologies underlying these conditions are relevant to the mechanisms of reproductive aging. The results of these epidemiologic studies are largely inconsistent, but conflicting findings may be explained by differences in population characteristics and endpoints studied, as well as potential confounding by dietary components and lifestyle factors.

1.4.1 Dairy and menopause timing

Perhaps the strongest evidence for a potential role of dairy in ovarian aging comes from a prospective study by Carwile et al, which evaluated age at natural menopause in relation to intakes of low-fat and high-fat dairy, skim and whole milk, dairy fat and protein, calcium, vitamin D, and lactose. (8) Participants included 46,059 premenopausal women enrolled in the prospective Nurses' Health Study (NHS) who were ages 30-55 at baseline in 1976. Intakes of the aforementioned exposures were measured using food frequency questionnaires (FFQs) every 4 years, and age at menopause was measured via self-report of cessation of menses on biennial questionnaires.

In multivariable analyses limited to premenopausal women less than 51 years of age, those who consumed >3 versus 0.1-1 servings/d of low-fat dairy foods experienced significantly lower risk of natural menopause occurring in the next month (Hazard ratio (HR): 0.86; 95% confidence interval (CI): 0.77-0.96; P-trend <0.001). Skim milk was similarly associated with lower risk among the women of the same age group (>6 servings/wk vs. 0-1 servings/mo HR: 0.93; 95% CI: 0.89-0.97; P-trend <0.001), while lactose, dairy fat, and dairy protein were modestly associated with lower risk.

Alternatively, intakes of high-fat dairy and whole milk were not associated with risk and findings for all foods were null among women ≥51 years of age.

More recently, Purdue-Smithe et al evaluated intakes of vitamin D and calcium and risk of early menopause among 86,234 premenopausal women enrolled in the Nurses' Health Study II (NHS2). (60) Participants were 25-42 at baseline in 1989 and responded to biennial questionnaires and FFQs administered every four years. Although researchers did not evaluate dairy foods directly, they observed that vitamin D intake from food sources, particularly dairy sources, was associated with significant lower risk of early menopause (dairy vitamin D Q5 vs. Q1 HR: 0.85; 95% CI: 0.74-0.98) after adjustment for risk factors of early menopause. Calcium intake from dairy sources was similarly associated with lower risk of early menopause (Q5 vs. Q1 HR: 0.87; 95% CI: 0.75-1.00; P-trend=0.03).

A third study prospectively evaluated the relationship of total dairy intake and age at natural menopause among 5110 premenopausal women of the European Investigation into Cancer and Nutrition (EPIC)-Heidelberg who were at least 35 years at baseline.(131) Total dairy intake was measured using a baseline FFQ, and self-reported age at menopause was defined as 12 consecutive months of amenorrhea not due to surgery. Results of multivariable analyses indicated no significant association of total dairy intake and age at menopause comparing the lowest versus the highest quartile (HR: 1.04; 95% CI: 0.86-1.27; P-trend = 0.998).

1.4.2 Dairy and other endpoints

Information from additional studies of dairy and other endpoints related to ovarian function such as AFC, POF, and infertility may also be relevant to understanding the relation of dairy and ovarian aging, although most of these studies were conducted among clinical populations, and it is unclear whether the physiologic mechanisms that underlie these conditions are similar.

1.4.2.1 Antral follicle count

Two studies have evaluated AFC in relation to dairy protein consumption. (132,133) A recent cross-sectional study by Souter et al reported an inverse association of dairy protein intake and AFC among women aged 18-45 years who were seeking treatment for infertility. (132) After adjusting for potential confounders, women in the highest quintile (range = 5.24-9.27% of energy) of dairy protein intake had 14.4% (95% CI: 3.9%-23.7%; $P<0.01$) lower AFC as compared to women in the lowest quintile (range = 0-2.31% of energy). These findings are consistent with a randomized trial of cynomolgus macaques, an established model for menopause in humans. (133) In this trial, 61 monkeys were randomly assigned to either a casein-lactalbulmin (dairy protein-based) diet or a eucaloric soy protein diet. After 32 months of the experimental diets, the monkeys fed the casein-lactalbumin diet had significantly fewer primordial, primary and secondary follicles ($P<0.05$), as compared to those in the soy protein diet arm.

1.4.2.2 Premature ovarian failure

To our knowledge, only one study has evaluated dairy intake in relation to POF. (134) In this small (n=160) case-control study, researchers observed no association of total dairy intake and POF (5.3 versus 5.6 times/week for cases and controls, respectively; P=0.5).

1.4.2.3 Infertility and anovulation

Studies of infertility and anovulation in relation to dairy consumption may also be relevant to ovarian aging. In a recent prospective study by Chavarro et al, researchers examined intakes of dairy foods and anovulatory infertility among 18,555 premenopausal women enrolled in the NHS2. (11) Interestingly, intake of low-fat dairy foods was associated with increased risk of anovulatory infertility (≥2 vs. ≤1 servings/d Relative risk (RR): 1.85; 95% CI: 1.24-2.77; P-trend=.002), whereas intake of high-fat dairy was associated with lower risk (≥1 vs. ≤1 servings/d RR: 0.73; 95% CI: 0.52-1.01; P-trend=0.01). The positive association of low-fat dairy with anovulatory infertility appears to be driven, at least in part, by yogurt intake; according to their findings, for each additional serving/d, risk of anovulatory infertility increased by 34% (95% CI: 1.02-1.74; P-trend=0.03). Likewise, the inverse association observed for high-fat dairy appears to be driven by intake of whole milk (RR for each 1 serving/d increase: 0.46; 95% CI: 0.25-0.84; P-trend=0.01). Estimates for both high and low-fat dairy foods were stronger among women older than 32 years, and among women with clinical manifestations of PCOS.

In another prospective study, Afeiche et al found that among women older than 35 years, total dairy intake was positively associated with live birth rate (quintile 1 live birth rate=16% versus quintile 4 live birth rate=53%; P=0.02), but was unrelated to probability of pregnancy or implantation. (135) An additional case-control study of female infertility (any cause), reported that women who drank >3 glasses/d of milk (fat content not specified) experienced 70% (95% CI: 0.1-0.7) lower odds of infertility, as compared to those who consumed no milk among agricultural workers in Wisconsin. (136)

In line with the findings of Chavarro et al, a small prospective study by Kim et al also reported a positive association of yogurt intake and sporadic anovulation among 259 healthy premenopausal women 18-44 years. (137) In this study, women who consumed >0 servings/d of yogurt experienced more than 2-fold risk of sporadic anovulation as compared to women who consumed no yogurt (RR=2.1; 95% CI=1.2-3.7). However, researchers also observed increased risk of anovulation among women who consumed cream, whereas intake of low-fat dairy, high-fat dairy, and other dairy foods were not associated with risk.

1.4.3 Synthesis of the evidence

Although the results of these epidemiologic studies are largely inconsistent, a number of potential contributing factors may explain, at least in part, the differences in findings. First, it is important to note that most of these studies evaluated different endpoints. It is currently unclear whether dairy may influence the underlying pathophysiologies of POF, infertility, and anovulation in a similar manner to ovarian aging and menopause timing. For example, POF represents an extreme end of the ovarian

aging spectrum and is strongly related to autoimmunity and genetic factors.(6) Risk of conditions like POF may thus be less modifiable than menopause timing in healthy premenopausal women.

The opposite directions of findings for high and low-fat dairy in relation to menopause timing and infertility and anovulation also raise questions about the potential influence of PCOS. PCOS is characterized by proliferation of small antral follicles, chronic anovulation and infertility. (138,139) Evidence suggests that women with PCOS have higher AMH levels due to increased antral follicle proliferation. (140,141) Consistent with the mechanisms involved in ovarian aging, some epidemiologic studies have observed that women with PCOS experience slower decline in AMH levels throughout the reproductive years, (142–144) and later age at menopause. (145)

~75-80% of women exhibit hyperandrogenism-related characteristics of PCOS including hirsutism and overweight/obesity, whereas ~20% are asymptomatic except for demonstrated subfertility due to chronic anovulation. (146) Because the diagnostic criteria for PCOS vary and depend largely upon the exhibition of phenotypic characteristics, (146) it stands to reason that the true prevalence of PCOS in the general population and especially in populations seeking treatment for infertility is likely higher than what is generally reported. At this time, studies of diet – particularly dairy intake – and PCOS are scarce, making it difficult to postulate how dairy, PCOS, and menopause timing may be interrelated. However, observations of stronger associations for specific dairy foods and anovulatory infertility among women with PCOS in the Chavarro et al study suggest that perhaps low-fat dairy is positively associated with risk of PCOS and high-fat dairy is associated with lower risk. (11) In light of evidence suggesting a relation

of PCOS and menopause timing, future studies evaluating dairy and menopause timing should carefully consider potential confounding and mediation by PCOS.

Second, it is unclear whether clinical endpoints are relevant and generalizable to healthy populations of premenopausal women. Epidemiologic studies evaluating menopausal timing with respect to dairy intake were conducted among healthy premenopausal women, whereas many of the studies evaluating other endpoints were conducted among women seeking treatment for infertility. As highlighted above, the potential influence of confounding by PCOS, especially in populations seeking treatment for infertility is high and warrants additional examination of the relationship of dairy and PCOS.

Third, many of the studies reviewed above evaluated different characteristics of dairy foods, which may have important implications regarding potential physiologic mechanisms. For instance, studies that separately evaluated high-fat versus low-fat dairy reported significant associations with their respective outcomes, (8,11) whereas studies evaluating only total dairy intake were null. (131) Although Greenlee et al observed a significant inverse association of total milk intake and infertility, their study population likely consumed mostly whole milk because the women who comprised the study sample were agricultural and dairy farmers. (136) The discordant findings of studies evaluating total dairy versus high and low-fat dairy may be explained by differences in hormonal mechanisms involving estradiol, progesterone, and androgen precursors in milk and their relative bioavailability contingent upon fat content.

The inverse associations of dairy protein intake and antral follicle count observed by 2 studies suggest that dairy protein, specifically, is associated with increased rate of

ovarian aging, (133,135) although it is currently unclear whether crude intake of dairy protein or relative intake of dairy protein versus other types of protein are more relevant. For example, in the Appt et al trial, monkeys in the casein-lactalbumin group were not fed dairy products per se, but rather isolated dairy protein. (133) Because there was no true control group of monkeys who adhered to a "normal" diet, results indicate only that the soy protein versus casein-lactalbumin diet is protective against ovarian aging in monkeys; it is unknown how a casein-lactalbumin diet would perform in relation to a "typical" diet. Given recent findings from one study that vegetable protein intake was associated with lower risk of early menopause, but animal protein was not, (61) it seems likely that the Appt findings may be attributed to the relative comparison of dairy versus soy protein.

Finally, Carwile et al's observation of a significant inverse associations of low-fat dairy and skim milk intake with age at menopause in women younger than 51 years of age, but not older women, suggests that a window of vulnerability may exist. (8) Nagel et al did not stratify estimates for total dairy and menopause according to age, presumably due to the lower power of their analysis. (131) If age does indeed modify the dairy-menopause timing relation, then failure to evaluate age-specific associations may partly explain differences in findings of these two studies.

As a whole, epidemiologic studies conducted to date raise a number of interesting questions and issues for consideration in future research. First, because observed associations for dairy and menopause timing appear to be modest, large prospective studies are necessary to ensure adequate statistical power for overall and stratified analyses. Second, to increase the generalizability of findings, future studies should

evaluate dairy and ovarian aging among populations of healthy premenopausal women, rather than limiting investigations to clinical populations. Third, because the relation of dairy, PCOS, and ovarian aging remains unclear, researchers should carefully consider the potential influence of PCOS on the dairy-ovarian aging relation. Finally, it appears to be important to separately evaluate high and low-fat dairy intake, as well as individual dairy constituents.

1.5 Conclusion

While laboratory evidence supports a plausible role for dairy in delaying ovarian aging, the epidemiologic evidence remains limited at this time. Future prospective studies that address the methodologic limitations of prior studies will be better equipped to answer the following questions:

- How are specific dairy nutrients (i.e., vitamin D, calcium, protein, fat, and lactose) related to ovarian aging?
- Does dairy intake in childhood, adolescence or early adulthood differentially influence rate of ovarian aging compared to intake in later reproductive years?
- How is dairy intake, PCOS, and ovarian aging interrelated?

Answers to these questions will provide a clearer picture of how dairy intake may be related to ovarian aging and through what mechanisms dairy may influence reproductive lifespan.

CHAPTER 2

VITAMIN D AND CALCIUM INTAKE AND RISK OF EARLY MENOPAUSE

2.1 Abstract

Early menopause, defined as the cessation of ovarian function before the age of 45, affects roughly 10% of women and is associated with higher risk for cardiovascular disease, osteoporosis and other conditions. Few modifiable risk factors for early menopause have been identified, but emerging data suggest that high vitamin D intake may reduce risk. We evaluated how intakes of vitamin D and calcium are associated with incidence of early menopause in the prospective Nurses' Health Study II. Intakes of vitamin D and calcium from foods and supplements were measured every 4 years by food frequency questionnaire. Cases of incident early menopause were identified from amongst all participants who were premenopausal at baseline in 1991; over 1.13 million person-years, 2,041 women reported natural menopause before age 45. We used Cox proportional hazards regression to evaluate relations between intakes of vitamin D and calcium and incident early menopause, accounting for potential confounding factors. After adjusting for age, smoking, and other factors, women with the highest intake of dietary vitamin D (quintile median=528 IU/d) had a significant 17% lower risk of early menopause compared to those with the lowest intake (quintile median=148 IU/d; HR: 0.83; 95% CI=0.72, 0.95; P-trend=0.03). Dietary calcium intake in the highest quintile (median=1,246 mg/d) versus the lowest (median=556 mg/d) was associated with borderline significant lower risk of early menopause (HR: 0.87; 95% CI: 0.76, 1.00; P-trend=0.03). Associations were stronger for vitamin D and calcium from dairy sources

than from non-dairy dietary sources, whereas high supplement use was not associated with lower risk. Findings suggest that high intake of dietary vitamin D and calcium may be modestly associated with lower risk of early menopause. Further studies evaluating 25-hydroxyvitamin D levels, other dairy constituents and early menopause are warranted.

2.2 Introduction

Early menopause, defined as the cessation of ovarian function before the age of 45, affects roughly 10% of women in Western populations. (1) Current research suggests that women who experience early menopause are at increased risk for premature mortality and cognitive decline, osteoporosis, and cardiovascular disease, among other adverse health outcomes. (2–5) Women commonly experience an ebb in fertility during the 10 years leading up to natural menopause; for women who have early menopause, this may have substantial financial and psychological consequences for family planning, particularly as women increasingly delay childbearing into the later reproductive years. (1,6) Genetic factors do not fully account for the age at which menopause occurs, and emerging research suggests that modifiable lifestyle factors such as diet may play an important role in ovarian aging. (8,9,120,121,131,147–149)

Calcium and vitamin D have been implicated in several gynecologic and reproductive conditions including polycystic ovary syndrome, endometriosis, and premenstrual syndrome, and appear to play a role in fertility. (13,63,150–153) Laboratory evidence suggests that the ovary is a target organ for $1,25(OH)_2D_3$, the active metabolite of vitamin D_3, and that vitamin D receptors (VDR) are expressed in reproductive tissues including the ovary. (154,155) Recently, one group observed that plasma 25-hydroxyvitamin D (25(OH)D) levels were positively associated with ovarian reserve, providing further evidence of a protective role of vitamin D on ovarian aging. (156)

Despite biologic plausibility, to our knowledge, no epidemiologic studies have specifically investigated the relation of vitamin D and calcium with incident early menopause. One recent prospective study of total vitamin D and calcium intake and

overall age at natural menopause among premenopausal women enrolled in the Nurses' Health Study (NHS) found that high intake of low-fat dairy foods, but neither total calcium nor total vitamin D intake were associated with later menopause. (8) Importantly, because the mean age at the beginning of follow-up for these women was 51.5, the relation of vitamin D and calcium intake and risk of early menopause (<45 years old) remains unclear. Further, this study did not distinguish calcium and vitamin D from dietary versus supplemental sources, which has proven to be an important distinction in prior studies of reproductive-related conditions and other outcomes. (13,157,158)

The aim of this study was to examine the relation of intakes of vitamin D and calcium from supplemental, dietary, dairy, and non-dairy dietary sources and subsequent risk of early menopause in the prospective Nurses' Health Study II (NHS2). We hypothesized that among participants of the NHS2, total intakes of vitamin D and calcium would be inversely associated with early menopause. We similarly hypothesized that vitamin D and calcium intake from supplements, food, and dairy would each be inversely associated with early menopause.

2.3 Subjects and Methods

The NHS2 is a prospective study of 116,430 female U.S. registered nurses who were 25-42 years old in 1989 when they responded to a mailed baseline questionnaire. Information regarding lifestyle behaviors and medical conditions are collected through biennial questionnaires, for which the follow-up rate for each cycle has been at least 89%. The study protocol was approved by the Institutional Review Board at Brigham and Women's Hospital in Boston, MA.

2.3.1 Assessment of early menopause

On the 1989 baseline questionnaire, nurses were asked if their periods had ceased permanently with the following response options: 1) No: Premenopausal; 2) Yes: No menstrual periods; 3) Yes: had menopause but now have periods induced by hormones; and 4) Not sure; (e.g., started hormones prior to cessation of periods). Nurses who indicated that their periods had ceased were then asked the following questions: 1) At what age did your periods cease? (open response); and 2) For what reason did your periods cease? (response options were surgery; radiation or chemotherapy; and natural). Additionally, women were asked about their current and past use of replacement sex hormones. These questions were then repeated on all subsequent questionnaires. Age at natural menopause was defined as age after 12 consecutive months of amenorrhea not due to radiation, chemotherapy or surgery. A small number of women reported being postmenopausal on one questionnaire and then subsequently reported being premenopausal. For these women, we defined age at menopause as age after which periods were absent for 12 months or more, and then confirmed that this status persisted for at least 3 consecutive questionnaires.

Because we were interested in prospectively evaluating the relationship of vitamin D and calcium with incident early menopause, participants were eligible for inclusion in our study if they indicated being premenopausal and reported no age at menopause on the baseline 1989 questionnaire (n = 108,812). We further excluded women who did not respond to or who reported implausible caloric intake (<500 or ≥3,500 kcal/d) on the 1991 food-frequency questionnaire (FFQ) (n = 21,904), were diagnosed with cancer before 1991 (n = 391), or whose date of menopause was before their return date of the

1991 FFQ (n = 283). After baseline exclusions, 86,234 women remained in the study sample.

Women were then followed prospectively until 2011 for self-report of the cessation of menses, as defined above, or first report of hysterectomy, bi-lateral or unilateral oophorectomy, cancer (not including non-melanoma skin cancer), loss to follow-up, or death. We identified cases of early menopause as women who reported natural menopause before the age of 45.

2.3.2 Dietary assessment

Nurses completed validated semi-quantitative FFQs in 1991, 1995, 1999, 2003, 2007, and 2011, which assessed their average intake of 131 foods, beverages, and supplements over the preceding year. (159–161) Each questionnaire asked participants to estimate, on average, how often they consumed specific foods and beverages. Participants reported their consumption by indicating one of nine frequency categories for each food and beverage (i.e., <1 serving/month, 1-3 servings/month, 1, 2-4, 5-6 servings/wk, and 1, 2-3, 4-5, and ≥6 servings/d). Calcium intake from food sources was estimated by summing calcium content per 1 serving of each food and beverage (i.e., skim, low-fat, and whole milk, yogurt, hard cheese, cottage cheese, spinach, etc.) and multiplying it by the frequency of consumption. Intakes of vitamin D, vegetable protein, and alcohol were derived similarly. We calculated percentage of total calories from vegetable protein by multiplying grams of vegetable protein by 4 kcal/g and then dividing by total kcal.

Nurses were queried about average use and dosage of multivitamins, calcium and vitamin D supplements every two years on FFQs or biennial questionnaires, from which

we estimated intakes of each nutrient from supplement sources. Total vitamin D and calcium intakes were then estimated by summing dietary intakes and supplemental intakes of each nutrient. Intakes of all nutrients were adjusted for total energy using the residual method. (162)

The validity of the FFQ was assessed by a comparison to 1-week diet records among a random subset (n = 100) of women in the Nurses' Health Study, a comparable population of female health professionals. The de-attenuated Pearson correlation coefficient comparing the two methods for calcium intake was 0.75. (159)

2.3.3 Assessment of covariates

Information regarding age, height, ethnicity, maternal and paternal education level, and age at menarche was collected at baseline in 1989. Updated information on weight, parity, oral contraceptive use, breastfeeding, hormone therapy use, and smoking were collected biennially throughout follow-up. Baseline height and updated weight were used to calculate updated body mass index (BMI) as weight (kg)/ height $(m)^2$ for each questionnaire cycle. Physical activity was assessed in 1991, 1997, 2001, 2005, and 2009 using nurses' responses to questions regarding average time spent per week participating in specific activities (i.e., walking, running, biking, etc.), from which we calculated metabolic equivalent task (MET)-hours per week. (163) For covariates with missing data, we assigned missing values to a missing indicator category. Results from analyses restricted to women with complete covariate data (n = 1,956) were identical; we have thus presented results from analyses using missing indicator categories to maximize statistical power.

2.3.4 Statistical analysis

We divided participants into quintiles of intake of total (foods + supplements), dietary (foods only), and dairy (dairy foods only) vitamin D and calcium according to the distribution of the NHS2 population. For supplemental vitamin D and calcium, we categorized participants according to their specified dosage (0, 1-599, and ≥600 IU/day for vitamin D; 0, 1-399, 400-899, and ≥900 for calcium), with non-users serving as the referent group. We additionally dichotomized participants according to the adequacy of their total vitamin D and calcium intake based on current Recommended Daily Allowances (RDA) (i.e., 600 IU/day for vitamin D; 1,000 mg/day for calcium) in order to aid in the interpretability of analyses. Baseline characteristics of our study sample according to total calcium and vitamin D intake were assessed using age-adjusted generalized linear models.

For our primary analyses, we used Cox proportional hazards regression to estimate age-adjusted and multivariable hazard ratios (HR) for early menopause by level of vitamin D and calcium intake. Tests for linear trend were conducted by modeling the median of each category as a continuous variable. For each participant, accrual of follow-up (in months) began on the date of return of the 1991 questionnaire and continued until menopause, first report of hysterectomy, bi-lateral or unilateral oophorectomy, cancer (not including non-melanoma skin cancer), loss to follow-up, or death, whichever occurred first. Analyses were stratified on age (in months) and questionnaire cycle.

For each nutrient, we modeled timing of intake in three ways: 1) baseline (1991) intake only; 2) simple updating of intake every 2 years; and 3) cumulative average intake. Cumulative average values for each exposure were calculated as mean intakes estimated

from all FFQs up to and including the cycle prior to menopause. Results from these three methods were similar, and because the cumulative average method is suggested to best represent long-term dietary intake by reducing misclassification due to within-person variation, we have presented only the results from cumulative average models. (164)

In addition to analyses adjusting only for age, we fit a full multivariable model adjusting for variables including age, pack-years of smoking, BMI, parity, lifetime duration of breastfeeding, age at menarche, physical activity, percentage of total calories from vegetable protein, and alcohol intake (MV1). Because none of the covariates tested were associated with a >10% change in exposure hazard ratios, covariate selection was based on factors identified a priori (i.e., age, smoking, BMI, parity, age at menarche, and physical activity) and factors associated with early menopause in our population (i.e., duration of breastfeeding and intakes of alcohol and vegetable protein). To account for the high correlation of vitamin D and calcium intakes, we mutually adjusted vitamin D for calcium and vice-versa, and supplemental intake for dietary intake and vice-versa in a second multivariable model.

To investigate potential variation in the associations by timing of exposure, we then evaluated whether vitamin D and calcium intake at age 35 versus at age 40 were differently associated with early menopause using logistic regression. Because not all participants completed questionnaires at exactly ages 35 or 40, dietary exposures and covariates on the questionnaire completed closest in time before age 35 and 40 were used. These analyses were restricted to women for whom diet data were available at both ages 35 and 40, and evaluated risk of early menopause from age 40 to <45 years (n = 534).

Because vitamin D is sequestered in adipose tissue (165) and BMI might plausibly behave as an effect modifier, we additionally tested the multiplicative interaction of BMI (3 categories) and dietary vitamin D (continuous quintile medians) on risk of early menopause using cross-product terms. We also conducted a number of sensitivity analyses to evaluate the stability of the estimates. First, in order to ensure the adequacy of our control for confounding by smoking, we restricted our sample to never smokers (n for analysis = 1,533 cases). Second, to evaluate the degree to which misclassification of early menopause may have occurred in our study, we additionally conducted analyses excluding non-cases who experienced menopause before age 48. We were also concerned that conditions indicated for hysterectomy that are also related to vitamin D and calcium intake may selectively exclude non-cases with low intake of these nutrients. To evaluate this potential selection bias, we conducted a sensitivity analysis censoring at date of laparoscopy-confirmed endometriosis and ultrasound-confirmed uterine fibroid diagnosis (n for analysis = 1,996 cases).

All statistical analyses were conducted with SAS v9.4 software (SAS Institute Inc., Cary, NC). We used two-sided statistical tests performed at the 0.05 significance level for all analyses.

2.4 Results

Over 1.13 million person-years of follow-up, 2,041 women experienced incident early menopause. Baseline age-adjusted descriptive statistics according to quintile of total vitamin D and calcium are presented in Table 2.1. On average, women who had the

highest intakes of calcium and vitamin D were younger, more physically active, had lower BMI, drank less alcohol and were less likely to be current smokers as compared to women with the lowest intakes.

Results from analyses of vitamin D intake are presented in Table 2.2. In age-adjusted analyses, total vitamin D intake was not associated with early menopause (HR: 0.95; 95% CI: 0.83, 1.09; P-trend = 0.80). Supplemental vitamin D intake ≥600 IU/d was also not associated with risk (HR: 1.30; 95% CI: 0.94, 1.78; P-trend = 0.23), while intakes of vitamin D from dietary and dairy sources were associated with lower risk of early menopause (HR: 0.79; 95% CI: 0.69, 0.91; P-trend <0.01 and HR: 0.82; 95% CI: 0.71, 0.94; P-trend = 0.02, respectively). For reference, this level of vitamin D intake from dairy sources corresponds to roughly 2.5 8-ounce servings of vitamin D-fortified milk per day. Intake of non-dairy dietary vitamin D was not associated with risk of early menopause (Q5 vs. Q1 HR: 0.94; 95% CI: 0.81, 1.07; P-trend = 0.32).

After further adjusting for risk factors for early menopause, estimates were similar to those of age-adjusted models. For example, in our first multivariable model (MV1) controlling for age, BMI, smoking and other risk factors, total vitamin D intake in the highest versus lowest quintile was not associated with risk of early menopause (Q5 vs. Q1 HR: 0.99; 95% CI: 0.86, 1.13; P-trend = 0.76). Women with dietary vitamin D intake in the highest versus the lowest quintile were 17% (95% CI: 0.72, 0.95; P-trend = 0.03) less likely to experience early menopause. To evaluate to whether this association may be driven by intake of these nutrients from dairy foods, we ran MV1 separately evaluating dairy versus non-dairy dietary vitamin D. The HR comparing the highest versus lowest quintiles of dairy vitamin D intake was 0.85 (95% CI: 0.74, 0.98; P-trend = 0.06),

whereas non-dairy dietary vitamin D was not associated with risk (Q5 vs. Q1 HR: 0.97; 95% CI: 0.84, 1.12; P-trend = 0.55). Vitamin D supplement use was also not associated with increased risk of early menopause (P-trend = 0.10).

Results from analyses of calcium intake and early menopause are presented in Table 2.3. Age-adjusted and MV1 estimates for total, dietary, and dairy calcium intake were similar to those for vitamin D. For example, in MV1, dietary calcium intake in the highest quintile was associated with a borderline significant 13% lower risk of early menopause (95% CI: 0.76, 1.00; P-trend = 0.03) as compared to the lowest. High calcium intake from dairy sources was also associated with borderline significant lower risk (Q5 vs. Q1 HR: 0.87, 95% CI: 0.75, 1.00; P-trend = 0.03), while non-dairy dietary calcium intake was not associated with risk of early menopause (Q5 vs. Q1 HR: 1.01, 95% CI: 0.85, 1.20, P-trend = 0.99). Calcium supplement intake was positively associated with risk of early menopause (P-trend <0.01).

In an attempt to disentangle the individual associations of vitamin D and calcium with risk of early menopause, we mutually adjusted corresponding sources of vitamin D and calcium in a second multivariable model. After adjustment, estimates were similar while confidence intervals were wider and no longer statistically significant (complete results not shown). For example, in this model, calcium intake from dairy foods in quintile 5 versus quintile 1 was associated with a non-significant 12% decreased risk of early menopause (HR: 0.88; 95% CI: 0.67, 1.14; P-trend = 0.29). The association for vitamin D intake from dairy foods was also attenuated (Q5 vs. Q1 HR: 0.96; 95% CI: 0.74, 1.25; P-trend = 0.94)

Results from MV1 logistic regression models assessing intakes of vitamin D and calcium from dietary and supplemental sources at ages 35 and 40 are presented in Table 2.4. At age 35, intake of supplemental vitamin D ≥600 IU/d compared to non-supplement users was associated with higher risk (OR: 1.93; 95% CI: 1.15, 3.22), while calcium supplement use was not associated with risk of early menopause. Intakes of dietary vitamin D and calcium were both inversely associated with risk of early menopause. Similarly, at age 40, intakes of both dietary vitamin D and calcium were inversely associated with early menopause. Supplemental vitamin D intake was not associated with risk, whereas high intake of supplemental calcium was associated with increased risk (≥900 mg/d vs. non-users: OR: 1.60; 95% CI: 1.19, 2.17; P-trend = 0.02)

Results from analyses among never smokers were similar but slightly stronger in magnitude than in main analyses (data not shown). The likelihood ratio test comparing models with and without total vitamin D-BMI multiplicative interaction terms was not statistically significant (P-interaction = 0.22). BMI also did not modify the dietary vitamin D-early menopause relation (P-interaction = 0.41). Estimates from analyses excluding non-cases with menopause occurring before age 48, and analyses censoring at diagnosis of endometriosis and uterine fibroids were similar to estimates from main analyses (data not shown). Tests of proportional hazards were not statistically significant, indicating that the proportional hazards assumption was met.

2.5 Discussion

In this prospective study, we found high intakes of vitamin D and calcium from food sources to be modestly associated with lower risk of early menopause. Contrarily, supplemental vitamin D was not associated with risk of early menopause and supplemental calcium was positively associated with early menopause.

The inverse associations for vitamin D and calcium from food sources appear to be driven largely by dairy sources of these nutrients, and mutual adjustment of vitamin D for calcium and vice-versa attenuated findings for each. While we are aware of no prior studies that have assessed dairy vitamin D and calcium specifically, Carwile et al observed that low-fat dairy, but not high-fat dairy intake, was associated with a later age of menopause, among women <51 years of age. (8) A similar association was reported in an abstract from the Japan Nurses' Health Study, which observed an inverse association of milk and dairy consumption on risk of early menopause; however, specific details about exposure and covariate measurement in this unpublished study are unavailable. (149) In contrast, dairy consumption was not associated with menopausal timing in a study within the European Prospective Investigation into Cancer and Nutrition.

It is notable that Carwile et al, similarly did not find total vitamin D or calcium intake to be related to age at menopause. (8) Their study, however, did not separately evaluate dietary versus supplemental sources of these nutrients, which may have attenuated associations for vitamin D from foods. In our study, high doses of supplemental calcium were associated with higher risk of early menopause. The observed higher risk among supplement users was unexpected, and we postulate that these women may have experienced conditions related to sex steroid hormone levels prior to early

menopause for which their doctors indicated a calcium supplement, such as autoimmune diseases or family history of osteoporosis.

To further address this potential bias, we also conducted a post hoc analysis (n for analysis = 1,757 cases) excluding women with conditions potentially related to both vitamin D and supplement use (e.g. lupus, multiple sclerosis, rheumatoid arthritis, low bone density, hip fracture, and osteoporosis). Estimates from these analyses were essentially identical to main analyses. We also considered the possibility that women were taking multivitamins containing supplemental vitamin D and calcium to treat perimenopause symptoms prior to early menopause. Nevertheless, after further controlling for multivitamin use, the positive association for supplemental calcium intake persisted.

The observed inverse association of dairy calcium and the borderline significant inverse association of dairy vitamin D specifically suggest that other constituents of dairy products may also influence menopause timing. Bovine milk is a rich source of steroid hormones - particularly progesterone; for example, whole milk contains 10 μg of progesterone per liter. (67) Milk consumption has also been shown to increase levels of plasma estradiol (86) and insulin-like growth factor (IGF-I) (89,90,92) in prior epidemiologic studies. It is therefore biologically plausible that these components, which are highly correlated with intakes of vitamin D and calcium in dairy, also contribute to the inverse associations observed. Studies of dairy foods and dairy constituents, such as phosphorus, potassium, and vitamin B-12, and early menopause are thus warranted.

Given that vitamin D and calcium are typically present together in specific foods, the attenuation of the estimates for each nutrient after mutual adjustment is likely due to

the collinearity of vitamin D and calcium. In our population, the Pearson correlation coefficient for dairy vitamin D and calcium in this population was 0.86 ($P<0.001$). It is likely that vitamin D and calcium each have individual contributions to the observed estimates, but separating out the associations for each nutrient is statistically impossible due to near perfect collinearity.

The observed inverse association for dietary vitamin D also may be due to a modest protective effect of vitamin D and calcium on ovarian aging. Several potential mechanisms supporting this hypothesis have been proposed – namely, the ability of vitamin D to modify mRNA expression of anti-Müllerian hormone, a glycoprotein secreted by granulosa cells during early follicle development that correlates with overall age at menopause and may play a role in regulating ovarian aging. (25,27,77,115,166) Because the majority of vitamin D is obtained via cutaneous synthesis during solar ultraviolet ray exposure, dietary and supplemental intake of vitamin D represents a small overall contribution to circulating plasma 25-hydroxyvitamin D (25(OH)D). (167) To better understand how vitamin D status is related to early menopause, future biomarker studies of anti-Müllerian hormone, plasma 25(OH)D levels and menopause timing are warranted.

Strengths of our study include a prospective design with 20 years of follow-up and a large sample size that allowed us to consider a variety of potential confounding variables. During this follow-up period, we were able to assess intakes of vitamin D and calcium five times and use cumulative averages of exposures, allowing us to capture within-person variation in diet and reduce misclassification. (164) Our study expanded upon prior studies by assessing early menopause specifically, rather than overall age at

menopause as an outcome. Menopausal timing follows a normal distribution, with a mean of 51 years of age. (6) In a prior study of other factors and menopausal timing, associations of nutrients varied across age strata (8), supporting the use of the binary early menopause outcome, rather than a continuous distribution of age at menopause with respect to evaluating risk factors.

Our study also has several limitations. First, because biochemical confirmation of age at menopause was not feasible in a study of this size, we relied upon self-reported menopausal status to determine timing of menopause. However, in a study of 6,591 women in the comparable NHS population, among women who were premenopausal in 1976 and reported having natural menopause on the 1978 questionnaire, 82% reported their age at menopause to within 1 year on the following two questionnaires. (168) Importantly, any misclassification of early menopause would most likely be biased towards null. Moreover, in a sensitivity analysis restricting non-cases to women with menopause after age 48, we found estimates to be identical, suggesting that our results are robust to misclassification of the outcome. Furthermore, while we cannot rule out the possibility of systematic over- or under-reporting of total energy, alcohol, and nutrients including vitamin D, calcium and vegetable protein, it is unlikely that reporting patterns for dietary variables would be related to case status. Misclassification across extreme categories is improbable and would not explain the observed inverse associations for intakes dietary and dairy vitamin D and calcium.

Because the NHS2 is a fairly heterogeneous population with regard to the dietary variables and other lifestyle factors related to early menopause, we anticipate that our results would be generalizable to other populations of premenopausal women. However,

given that the NHS2 is racially homogenous, our ability to assess differences in the vitamin D/calcium-early menopause relation across racial and ethnic groups is limited and our findings should be replicated in more racially diverse populations. It is also important to note that in the NHS2, dietary vitamin D intake in the highest category is higher than what has been reported in other population-based studies (169) and thus may not reflect typical intake in the U.S. Findings from our study suggest that vitamin D and calcium from food sources, particularly dairy, are modestly associated with lower risk of early menopause. Further studies examining risk of early menopause with regard to dairy constituents and plasma vitamin D levels are warranted.

Table 2.1 Age-adjusted characteristics of premenopausal women according to category of total vitamin D and calcium intake (intake from foods and supplements combined) at baseline (1991): Nurses' Health Study II, 1991[1]

	Total Vitamin D (Quintiles)					Total Calcium (Quintiles)				
Characteristic	Q1 (n=17000)	Q2 (n=17069)	Q3 (n=17438)	Q4 (n=17421)	Q5 (n=17306)	Q1 (n=17024)	Q2 (n=17333)	Q3 (n=17440)	Q4 (n=17476)	Q5 (n=16961)
Calcium intake,[2] mg/d	684 ± 2.7	842 ± 2.7	1024 ± 2.7	1156 ± 2.7	1364 ± 2.7	570	753	923	1163	1560
Vitamin D intake,[3] IU/d	128	217	317	473	742	214 ± 1.7	289 ± 1.7	370 ±1.7	459 ± 1.7	617 ± 1.7
Age,[4] y	36.1 ± 4.6	36.1 ± 4.6	35.9 ± 4.6	35.5 ± 4.6	35.3 ± 4.5	36.2 ± 4.6	35.9 ± 4.6	35.7 ± 4.6	35.5 ± 4.6	35.6 ± 4.7
BMI, kg/m^2	24.8 ± 0.04	24.6 ± 0.04	24.5 ± 0.04	24.4 ± 0.04	24.3 ± 0.04	24.6 ± 0.04	24.6 ± 0.04	24.6 ± 0.04	24.4 ± 0.04	24.4 ± 0.04
Age at menarche, y	12.4 ± 0.01	12.4 ± 0.01	12.4 ± 0.01	12.4 ± 0.01	12.4 ± 0.01	12.4 ± 0.01	12.4 ± 0.01	12.4 ± 0.01	12.4 ± 0.01	12.4 ± 0.01
Full-term pregnancies, *n*	1.6 ± 0.01	1.6 ± 0.01	1.6 ± 0.01	1.6 ± 0.01	1.5 ± 0.01	1.5 ± 0.01	1.6 ± 0.01	1.5 ± 0.01	1.6 ± 0.01	1.5 ± 0.01
Physical activity, MET-h/wk	20.6 ± 0.5	23.1 ± 0.50	24.9 ± 0.5	25.0 ± 0.5	27.5 ± 0.5	21.3 ± 0.5	23.2 ± 0.5	24.8 ± 0.5	24.9 ± 0.5	26.9 ± 0.5
Vegetable protein intake, % of total kcal	5.0 ± 0.01	5.1 ± 0.01	5.0 ± 0.01	5.0 ± 0.01	5.0 ± 0.01	4.8 ± 0.01	5.1 ± 0.01	5.2 ± 0.01	5.0 ± 0.01	4.9 ± 0.01
Alcohol intake, g/d	3.5 ± 0.05	3.3 ± 0.05	3.2 ± 0.05	3.1 ± 0.05	2.5 ± 0.05	3.5 ± 0.05	3.6 ± 0.05	3.3 ± 0.05	2.9 ± 0.05	2.5 ± 0.05
Ever used OCs, %	84.8	84.7	84.3	82.9	83.4	84.2	84.6	84.0	83.4	83.8
Current smoker, %	16.4	12.9	10.9	10.0	9.1	17.1	12.9	10.9	9.7	8.7

[1]Values are means ± SEs or percentages, unless otherwise indicated. All characteristics were calculated with the use of generalized linear models adjusted for the age of participants in 1991. MET-h, metabolic equivalent task hours; Q, quintile.

[2]Values reflect total calcium intake (mg/d) per quintile of total vitamin D and quintile medians for total calcium.

[3]Values reflect total vitamin D intake (IU/d) per quintile of total calcium and quintile medians for total vitamin D.

[4]Values are not age-adjusted. Values are means ± SDs.

Table 2.2 HRs (95% CIs) for early menopause by level of cumulatively averaged vitamin D intake: Nurses' Health Study II (1991-2011)[1]

	Median	Cases	Age-adjusted HR (95% CI)	Multivariable 1[2] HR (95% CI)
Total Vitamin D	(IU/d)			
Q1	145	438	1	1
Q2	241	392	0.86 (0.75, 0.99)	0.89 (0.78, 1.02)
Q3	341	419	0.90 (0.79, 1.03)	0.95 (0.83, 1.08)
Q4	474	399	0.89 (0.77, 1.02)	0.94 (0.82, 1.07)
Q5	695	393	0.95 (0.83, 1.09)	0.99 (0.86, 1.13)
P-trend			0.80	0.76
RDA (IU/d)				
<600	301	1711	1	1
≥600	721	330	1.02 (0.90, 1.15)	1.02 (0.91, 1.15)
Dietary Vitamin D				
Q1	148	441	1	1
Q2	232	424	0.94 (0.82, 1.07)	0.97 (0.85, 1.11)
Q3	301	375	0.81 (0.71, 0.93)	0.85 (0.74, 0.98)
Q4	383	441	0.96 (0.84, 1.09)	1.01 (0.88, 1.15)
Q5	528	360	0.79 (0.69, 0.91)	0.83 (0.72, 0.95)
P-trend			<0.01	0.03
Vitamin D from Dairy Sources				
Q1	25	436	1	1
Q2	62	403	0.88 (0.77, 1.01)	0.90 (0.79, 1.03)
Q3	101	399	0.86 (0.75, 0.99)	0.90 (0.78, 1.03)
Q4	153	419	0.88 (0.77, 1.01)	0.92 (0.80, 1.05)
Q5	254	384	0.82 (0.71, 0.94)	0.85 (0.74, 0.98)
P-trend			0.02	0.06
Non-Dairy Dietary Vitamin D				
Q1	46	436	1	1
Q2	75	421	0.96 (0.84, 1.10)	1.02 (0.89, 1.16)
Q3	101	401	0.93 (0.81, 1.07)	0.99 (0.86, 1.14)
Q4	135	397	0.93 (0.81, 1.06)	0.98 (0.86, 1.13)
Q5	196	386	0.94 (0.81, 1.07)	0.97 (0.84, 1.12)
P-trend			0.32	0.55
Supplemental Vitamin D (IU/d)				
0	0	899	1	1
1-599	209	1102	1.01 (0.92, 1.11)	1.04 (0.95, 1.15)
≥600	800	40	1.30 (0.94, 1.78)	1.29 (0.94, 1.77)
P-trend			0.23	0.10

[1]MET, metabolic equivalent task; Q, quintile; RDA, Recommended Daily Allowance.

[2]Multivariable Cox proportional hazards model adjusted for age, pack-years of smoking (0-10, 11-20, or ≥21), BMI [in kg/m^2 (<18.5, 18.5-<25, 25-<30, or ≥30)], age at menarche (continuous), parity (nulliparous, 1-2, or ≥3), breastfeeding duration (in months; continuous), physical activity (in continuous MET-h/wk), % of total calories from vegetable protein (quintiles 1-3, 4+5), and alcohol intake (<10, ≥10 g/d).

Table 2.3 HRs (95% CIs) for early menopause by level of cumulatively averaged calcium intake: Nurses' Health Study II (1991-2011)[1]

	Median	Cases	Age-adjusted HR (95% CI)	Multivariable 1[2] HR (95% CI)
Total Calcium	(mg/d)			
Q1	609	419	1	1
Q2	802	477	1.09 (0.95, 1.24)	1.16 (1.01, 1.32)
Q3	982	421	0.96 (0.84, 1.10)	1.04 (0.90, 1.19)
Q4	1205	337	0.79 (0.68, 0.91)	0.86 (0.74, 0.99)
Q5	1566	387	1.01 (0.88, 1.15)	1.09 (0.94, 1.25)
P-trend			0.11	0.60
RDA (mg/d)				
<1,000	771	1177	1	1
≥1,000	1270	864	0.89 (0.82, 0.97)	0.93 (0.85, 1.01)
Dietary Calcium				
Q1	556	426	1	1
Q2	705	414	0.91 (0.79, 1.04)	0.96 (0.84, 1.10)
Q3	832	437	0.95 (0.83, 1.09)	1.02 (0.89, 1.17)
Q4	987	390	0.83 (0.73, 0.96)	0.90 (0.78, 1.03)
Q5	1246	374	0.81 (0.70, 0.93)	0.87 (0.76, 1.00)
P-trend			<0.01	0.03
Calcium from Dairy Sources				
Q1	246	426	1	1
Q2	382	410	0.90 (0.79, 1.03)	0.94 (0.82, 1.08)
Q3	503	426	0.93 (0.81, 1.06)	0.97 (0.85, 1.11)
Q4	657	397	0.85 (0.74, 0.97)	0.90 (0.78, 1.03)
Q5	926	382	0.83 (0.72, 0.95)	0.87 (0.75, 1.00)
P-trend			<0.01	0.03
Non-Dairy Dietary Calcium				
Q1	235	415	1	1
Q2	280	430	1.02 (0.89, 1.17)	1.11 (0.96, 1.27)
Q3	312	398	0.92 (0.80, 1.06)	1.03 (0.89, 1.20)
Q4	347	421	0.98 (0.85, 1.12)	1.12 (0.96, 1.31)
Q5	410	377	0.89 (0.77, 1.02)	1.01 (0.85, 1.20)
P-trend			0.06	0.99
Supplemental Calcium (mg/d)				
0	0	1019	1	1
1-399	139	714	0.98 (0.88, 1.08)	1.01 (0.91, 1.12)
400-899	512	252	1.31 (1.14, 1.50)	1.36 (1.18, 1.56)
≥900	1015	56	0.99 (0.75, 1.29)	1.03 (0.78, 1.35)
P-trend			0.02	<0.01
Vitamin D or Calcium Supplement Use				
Non-user	772	N/A	1	1
Vitamin D only	247	N/A	0.99 (0.86, 1.14)	1.01 (0.87, 1.17)
Calcium only	127	N/A	1.06 (0.88, 1.28)	1.09 (0.90, 1.32)
Calcium + Vitamin D	895	N/A	1.04 (0.94, 1.16)	1.09 (0.98, 1.21)

[1]MET, metabolic equivalent task; Q, quintile; RDA, Recommended Daily Allowance.

[2]Multivariable Cox proportional hazards model adjusted for age, pack-years of smoking (0-10, 11-20, or ≥21), BMI [in kg/m^2 (<18.5, 18.5-<25, 25-<30, or ≥30)], age at menarche (continuous), parity (nulliparous, 1-2, or ≥3), breastfeeding duration (in months; continuous), physical activity (in continuous MET-h/wk), % of total calories from vegetable protein (quintiles 1-3, 4+5), and alcohol intake (<10, ≥10 g/d).

Table 2.4 Multivariable ORs (95% CIs) for early menopause vitamin D and calcium intakes assessed at ages 35 and 40: Nurses' Health Study II (1991-2011)[1-3]

	Intake Assessed at Age 35			Intake Assessed at Age 40		
	Median	Cases	OR (95% CI)	Median	Cases	OR (95% CI)
Dietary Vitamin D						
Q1	107	114	1	76	119	1
Q2	170	122	1.04 (0.80, 1.35)	137	96	0.76 (0.58, 1.00)
Q3	226	89	0.71 (0.54, 0.95)	190	107	0.89 (0.68, 1.17)
Q4	295	103	0.81 (0.62, 1.07)	254	108	0.86 (0.66, 1.13)
Q5	403	106	0.80 (0.61, 1.05)	363	104	0.82 (0.63, 1.08)
P-trend			0.04			0.38
Supplemental Vitamin D (IU/d)						
0	0	306	1	0	258	1
1-599	400	211	0.81 (0.68, 0.98)	228	266	1.10 (0.92, 1.31)
≥600	800	17	1.93 (1.15, 3.22)	800	10	0.89 (0.47, 1.70)
P-trend			0.33			0.67
Dietary Calcium						
Q1	527	106	1	509	104	1
Q2	680	93	0.77 (0.57, 1.02)	661	109	1.07 (0.81, 1.43)
Q3	814	113	0.87 (0.66, 1.14)	797	117	1.08 (0.82, 1.42)
Q4	988	108	0.76 (0.58, 1.01)	973	102	0.90 (0.68, 1.19)
Q5	1284	114	0.77 (0.59, 1.01)	1280	102	0.89 (0.67, 1.18)
P-trend			0.12			0.17
Supplemental Calcium (mg/d)						
0	0	353	1	0	286	1
1-399	162	117	0.89 (0.72, 1.10)	162	131	1.08 (0.88, 1.34)
400-899	500	44	1.07 (0.77, 1.48)	500	62	0.93 (0.70, 1.23)
≥900	1055	20	1.15 (0.72, 1.82)	1000	55	1.60 (1.19, 2.17)
P-trend			0.59			0.02
Vitamin D or Calcium Supplement						
Non-user	N/A	282	1	N/A	225	1
User	N/A	252	0.88 (0.74, 1.05)	N/A	309	1.08 (0.90, 1.29)

[1]MET, metabolic equivalent task; Q, quintile.

[2]Analysis limited to women with vitamin D and calcium intake assessed at both age 35 and age 40; case ascertainment limited to women with early menopause occurring after diet assessment at age 40.

[3] Multivariable Cox proportional hazards model adjusted for age, pack-years of smoking (0-10, 11-20, or ≥21), BMI [in kg/m^2 (<18.5, 18.5-<25, 25-<30, or ≥30)], age at menarche (continuous), parity (nulliparous, 1-2, or ≥3), breastfeeding duration (in months; continuous), physical activity (in continuous MET-h/wk), % of total calories from vegetable protein (quintiles 1-3, 4+5), and alcohol intake (<10, ≥10 g/d).

CHAPTER 3

TOTAL AND FREE 25-HYDROXYVITAMIN D AND VITAMIN D BINDING PROTEIN LEVELS AND RISK OF EARLY MENOPAUSE

3.1 Abstract

Early natural menopause, the cessation of ovarian function before age 45 y, is associated with higher risk of cardiovascular disease and other conditions. Dietary vitamin D intake has been associated with lower risk of early menopause; however, no prior studies have evaluated risk with regard to plasma 25-hydroxyvitamin D (25(OH)D) levels. We prospectively evaluated the association of total and free 25(OH)D and vitamin D binding protein (VDBP) levels with risk of early menopause in a case-control study nested within the Nurses' Health Study II (NHS2). We also considered associations of 25(OH)D and VDBP with levels of anti-Müllerian hormone (AMH). The NHS2 is a prospective cohort study of 116,430 nurses, aged 25-42 y at baseline (1989). Premenopausal plasma blood samples were collected between 1996-1999, from which total 25(OH)D and VDBP levels were measured and free 25(OH)D levels were calculated. Cases were women who experienced natural menopause between blood collection and age 45 y (n=328) and were matched 1:1 to controls experiencing menopause after age 48 y (n=328) by age and other factors. In multivariable conditional logistic models adjusting for matching factors, smoking, body mass index, and other factors, total and free 25(OH)D were not strongly associated with risk of early menopause. Odds ratios (OR) comparing the highest vs. lowest quartile was 1.04 (95% CI: 0.60, 1.81) for total 25(OH)D and 0.70 (95% CI: 0.41, 1.20) for free 25(OH)D. Plasma 25(OH)D was unrelated to AMH levels. VDBP was

positively associated with early menopause; the OR comparing the highest vs. lowest quartile of VDBP was 1.80 (95% CI: 1.09, 2.98). Our findings suggest that total and free 25(OH)D are not importantly related to risk of early menopause. VDBP may be associated with increased risk, but replication in more racially-diverse populations is warranted.

3.2 Introduction

Early menopause, which is the cessation of ovarian function before the age of 45 y, affects approximately 10% of women in Western populations. (1) Women who experience early menopause are at increased risk for premature mortality and cognitive decline, osteoporosis, and cardiovascular disease, as well as other adverse health outcomes. (2–5) Early menopause may also be problematic for couples trying to conceive, as female fertility declines drastically during the 10 years leading up to menopause. Couples unable to conceive as they wish may experience substantial financial and psychological consequences, particularly as women increasingly delay childbearing into the later reproductive years. (1,6) As such, it is important to identify modifiable lifestyle factors that may be related to early menopause risk, including diet.

Accelerated ovarian aging, the mechanism thought to underlie early menopause, is characterized by a decline in the quantity and quality of the ovarian follicle pool. (6) During the reproductive years, primordial follicle growth and transition to primary follicles are inhibited by anti-Müllerian hormone (AMH), a glycoprotein secreted by the granulosa cells of primary, secondary, and small antral follicles. Notably, a vitamin D response element has been identified in the promoter region of the AMH gene (75), suggesting a potential role of vitamin D in AMH secretion and thus follicle recruitment. In line with this hypothesis, laboratory studies have demonstrated that vitamin D upregulates AMH mRNA expression in both human prostate cells and granulosa cells of hen. (76,77)

Findings of a recent study in the Nurses' Health Study II (NHS2) cohort suggest that vitamin D intake from food sources, particularly dairy sources, is associated with

lower risk of early menopause. (60) Because vitamin D is obtained through dietary intake as well as sunlight exposure, levels of 25(OH)D, the primary circulating metabolite of vitamin D, is a better indicator of vitamin D status than dietary intake alone. To our knowledge, no prior studies have evaluated 25(OH)D levels and risk of early menopause and findings of epidemiologic studies evaluating 25(OH)D levels and AMH are conflicting. (115,166,170,171) One recent study found that AMH levels exhibit seasonal variation correlated with 25(OH)D levels and that vitamin D supplementation prevented a seasonal decline in AMH levels. (166) An additional study among U.S. women found 25(OH)D to be positively associated with AMH levels (115), while three more recent studies reported no association. (170,171)

It is also currently unclear whether measures of total versus free 25(OH)D levels may have different relationships with ovarian aging. At any given time, over 99% of vitamin D in the body is bound to vitamin D binding protein or albumin, leaving less than 1% unbound. (73) Because vitamin D binding protein levels influence the bioavailability of vitamin D, assessing total versus free 25(OH)D levels, as well as levels of vitamin D binding protein may be important for understanding the potential relation of vitamin D and early menopause. In fact, recent epidemiologic studies have observed that the free 25(OH)D fraction, as compared to total 25(OH)D, is more strongly associated with several health outcomes such as bone mineral density (172) and colorectal cancer. (173)

The aims of the present nested case-control study were thus to evaluate associations of total 25(OH)D, free 25(OH)D, and vitamin D binding protein levels (VDBP) and risk of early menopause among participants of the NHS2.

3.3 Subjects and Methods

The NHS2 is a prospective study of 116,429 female U.S. registered nurses who were 25-42 years old in 1989 when they responded to a mailed baseline questionnaire. Information regarding lifestyle behaviors and medical conditions are collected through biennial questionnaires, for which the follow-up rate for each cycle has been at least 89%. The study protocol was approved by the institutional review board at Brigham and Women's Hospital in Boston, MA.

3.3.1 Case and control ascertainment

On the 1989 baseline questionnaire, nurses were asked if their menstrual periods had ceased permanently and were provided the following response options: 1) No: Premenopausal; 2) Yes: No menstrual periods; 3) Yes: had menopause but now have periods induced by hormones; and 4) Not sure; (e.g., started hormones prior to cessation of periods). Nurses who indicated that their periods had ceased were then asked the following questions: 1) At what age did your periods cease? (open response); and 2) For what reason did your periods cease? (response options were surgery; radiation or chemotherapy; and natural). Women were also asked about their current and past use of menopausal hormone therapy (HT). Questions regarding menopausal status and use of HT were then repeated on all subsequent questionnaires.

A small number of women reported being postmenopausal on one questionnaire after a long interval of amenorrhea, only to have periods return again and report being premenopausal on a subsequent questionnaire. For these women, we defined age at menopause as age after which periods were absent for 12 mo or more, and then

confirmed that this status persisted for at least 3 consecutive questionnaires in order to reduce potential for misclassification of early menopause.

3.3.2 Blood sample collection

Participants of the NHS2 who had not previously been diagnosed with cancer (n=92,888) were invited to provide blood samples between 1996 and 1999. During this time period, participants were ages 32-54 y. Premenopausal women who were not pregnant and were not using oral contraceptives (OC) or menopausal HT, were asked to provide two samples during the span of one menstrual cycle. The first sample was to be collected during the follicular phase (days 3-5 of the menstrual cycle) and the second was to be collected during the luteal phase (7-9 days before the start of the next menses). Women who were using OCs or menopausal HT were asked to provide a single untimed sample, and women who reported irregular menstrual cycles were asked to collect a luteal phase sample 22 days after the last menses. To confirm timing and menstrual cycle phase of the samples, women completed a postcard at the start of their next menses. Upon receipt, blood samples were centrifuged, separated into plasma, buffy, and red blood cell components and then stored at ≤-130 °C in nitrogen freezers.

Among eligible women, 29,611 provided a sample and ~23,000 of these women were premenopausal at the time of blood draw. Women who provided samples were similar to the entire NHS2 cohort, having equivalent BMI (26 vs. 26 kg/m^2) and parity (1.9 vs. 1.9 children), and comparable proportions of ever smoking (34% vs. 36%) and history of OC use (86% vs. 88%), as well as other factors. (174)

Because we were interested in prospectively evaluating the relation of plasma 25(OH)D levels and risk of early menopause, we limited eligibility to women who experienced menopause after blood draw. In addition, our study sample was limited to women without a previous diagnosis of cancer (other than non-melanoma skin cancer), myocardial infarction, stroke, coronary artery bypass surgery, or percutaneous coronary intervention and women who had available plasma samples. Cases of early menopause were then defined as women reporting natural menopause (i.e., not due to surgery or chemotherapy) before the age of 45 y during follow-up. To reduce potential for misclassification of early menopause status, eligible controls were women who experienced menopause after age 48 y (Q3 of the interquartile range (IQR)).

Eligible cases (n=328) were then matched 1:1 to controls according to age at blood collection (within 4 mo), time of day of blood collection, month of collection (within 3 mo), sample type (luteal phase or untimed) and fasting status.

3.3.3 Laboratory assays

Plasma 25(OH)D, VDBP, albumin, and AMH were measured in the laboratory of Dr. Nader Rifai at Boston's Children's Hospital (Boston, MA). Immunoassays were used for measurement of plasma 25(OH)D (Immunodiagnostic Systems Inc., Fountain Hills, AZ) and plasma VDBP (R&D Systems (Minneapolis, MN)). The immunoassay used to measure plasma VDBP was a monoclonal antibody. Plasma AMH was measured using the pico AMH assay from ANSH Labs (Webster, TX).

Using measured values of plasma total 25(OH)D, VDBP, and albumin for each individual, we calculated each participants' plasma free 25(OH)D level according to the following equation (175):

$$Free\ 25(OH)D\ (pmol/L) = \frac{total\ 25(OH)D\ (nmol/L)}{1 + (6{\times}10^{3}{\times}\ albumin\ (g/L)) + (7{\times}10^{8}{\times}VDBP\ (\mu mol/L))}$$

To prevent potential exposure measurement error related to case status, laboratory personnel were blinded to case/control status for all assays. Samples were labeled by number and matched case-control sets were handled together, shipped in the same batch, and assayed in the same analytical run. Masked quality control samples were randomly interspersed among case-control samples and were analyzed in each batch. The coefficients of variation for biomarkers were 4.9% for total 25(OH)D, 7.2% for VDBP, and 8.6% for AMH.

3.3.4 Assessment of covariates

Variables used as matching factors, including age at blood collection, time of day of blood collection, month of collection, sample type (luteal phase or untimed), and fasting status were assessed at the time of blood draw. We also considered the following variables for inclusion in multivariable models based on previous studies of risk factors for early menopause: race/ethnicity, smoking, BMI, parity, physical activity, OC use and duration, duration of breastfeeding, alcohol intake, and vegetable protein intake. Height and race/ethnicity were assessed at baseline in 1989 and information regarding smoking status, weight (to calculate BMI), alcohol intake, fasting status, and menstrual cycle phase were collected at the time of blood draw by separate questionnaire. Updated information regarding smoking, weight, OC use, parity, and breastfeeding was assessed

on biennial questionnaires beginning in 1989. Physical activity was assessed in 1989, 1991, 1997, 2001, and 2005 using validated questionnaires. (163) Finally, validated food frequency questionnaires (FFQ) administered every 4 years were used to assess dietary intake of total calories, alcohol, and vegetable protein. (159–161) Intakes of nutrients were adjusted for total energy using the residual method. (162) For time-varying covariates, we modeled variables that corresponded to questionnaires closest in time to blood collection for each individual.

3.3.5 Statistical analysis

We used Chi-square and t-tests to compare characteristics of early menopause cases and controls at the time of blood draw and histogram and normality plots to assess the normality of biomarker data. Participants were divided into quartiles of total 25(OH)D, free 25(OH)D, and VDBP based on the distribution of these biomarkers in the control group. Likelihood ratio tests comparing nested models to were used to assess the global significance of each biomarker. We also categorized participants according to the following cutpoints: <50, 50-<75, and ≥75 nmol/L. (176) For analyses using continuous biomarker data, we identified and removed outliers using the Rosner extreme studentized deviate test and standardized continuous variables to aid in interpretability. Relations of total and free 25(OH)D with log(AMH) were evaluated using multivariable generalized linear models. We then back-calculated AMH geometric means and 95% confidence intervals (CI) according to each quartile of total and free 25(OH)D.

For each exposure, we used conditional logistic regression to calculate odds ratios (OR) and 95% CI adjusting for matching factors only. We then controlled for potential

confounders of the 25(OH)D-early menopause relation using multivariable conditional logistic regression. Because none of the covariates that were tested produced a change in exposure estimates >10%, covariates in multivariable models were selected if they were importantly associated with the outcome in our population.

To assess potential non-linear associations of each biomarker exposure with risk of early menopause, we additionally ran restricted cubic spline models. For these models, we specified four knots in order to evaluate the individual spline term contributions to the model fit and overall test for non-linearity.

We also conducted a number of stratified analyses to assess potential effect modification of relations of vitamin D with incident early menopause. To assess potential variation in associations according to age, we stratified our analyses by median age at blood draw (40 yrs). Second, because vitamin D is sequestered in adipose tissue (165), we assessed potential BMI effect modification by stratifying according to 2 categories of BMI (<25 vs. ≥ 25 kg/m^2). Third, to evaluate potential seasonal variation in the 25(OH)D-early menopause relation, we stratified our analyses by season of blood collection (winter/spring and summer/fall).

We also conducted sensitivity analyses to determine the robustness of estimates in primary analyses. Autoimmune conditions such as multiple sclerosis, rheumatoid arthritis, and lupus may be associated with lower 25(OH)D levels and earlier age at menopause. Accordingly, we conducted analyses excluding individuals diagnosed with these conditions at any point during follow-up. Similarly, we conducted analyses limited to women with luteal phase samples in order to address the potential impact of variation of biomarker measures due to menstrual cycle variability. We also conducted analyses

limited to never smokers to evaluate potential residual confounding due to misclassification of smoking amount among smokers. All analyses were conducted using SAS v9.4 software (SAS Institute Inc., Cary, NC). We used two-sided statistical tests performed at the 0.05 significance level for all analyses.

3.4 Results

Characteristics of cases and controls at the time of blood collection are presented in Table 3.1. At the time of blood collection, cases were more likely to identify as non-White and to smoke, and reported lower intakes of alcohol, supplemental calcium, and total vitamin D and calcium than controls.

In unadjusted analyses and analyses adjusting for important covariates, total 25(OH)D levels were not associated with risk (Table 3.2). For example, the OR comparing the first and fourth quartile of total 25(OH)D was 1.04 (95% CI: 0.60-1.81) in our MV1 model. Results for free 25(OH)D were suggestive of a lower risk at the highest levels, but confidence intervals were wide and results were not significant (Q4 vs. Q1 OR: 0.70; 95% CI: 0.41-1.20). High vitamin D binding protein was associated with increased risk, and results were stronger after adjustment for variables in MV1, with some evidence of a threshold of higher risk for quartiles 3 and 4 versus quartile 1 (Q3 vs. Q1 OR: 1.80; 95% CI: 1.10-2.95 and Q4 vs. Q1 OR: 1.80; 95% CI: 1.09-2.98). Results comparing women with total 25(OH)D levels of ≥75 versus those with <50 nmol/L were null (MV2 OR: 1.19; 95% CI: 0.57-2.46) (Table 3.4). For each exposure, results from restricted cubic spline models indicated no associations with early menopause – either linear or non-linear (*P* for all exposures > 0.10; complete results not shown).

We did not find that adjusted geometric means of AMH levels varied according to quartile of total (*P*=0.55) or free 25(OH)D levels (*P*=0.32) (Table 3.3). AMH geometric mean levels varied significantly across VDBP quartiles, with the lowest levels in Q3 and Q4 (*P*=0.04).

Findings of the primary analyses were largely unchanged in models evaluating effect modification and in sensitivity analyses. Estimates from analyses of total 25(OH)D stratified by median age at blood draw (< or ≥40 yrs) were consistent with findings from main analyses (data not shown). Estimates for total 25(OH)D stratified by BMI (underweight/normal and overweight/obese) were unstable due to small numbers (data not shown). Season (summer/fall vs. winter/spring) did not modify associations for total 25(OH)D or free 25(OH)D (*P*-interactions = 0.31 and 0.85, respectively). Estimates were also similar after restricting analyses to non-smokers (n=222 cases and 216 controls), women who provided timed blood samples (n=254 cases and 254 controls), and women without diagnoses of autoimmune conditions (n=321 cases and 319 controls). We also ran models using only 'super-normal controls' defined as women who experienced menopause at the population mean age of 51 years (n=328 cases and 63 controls). Estimates from these models were not materially different than those of our main analyses. For example, the MV1 OR for each 1 standard deviation increase in total 25(OH)D was 1.29 (95% CI: 0.87-1.91) and for free 25(OH)D was 1.00 (95% CI: 0.68-1.46).

3.5 Discussion

In this prospective study, we did not find vitamin D metabolite levels to be consistently or strongly associated with risk of early menopause, or with levels of plasma AMH, a marker of ovarian aging. Conversely, high versus low VDBP levels was associated with increased risk of early menopause and lower AMH levels.

To our knowledge, this is the first study to investigate the association of total and free 25(OH)D and vitamin D binding protein levels and risk of early menopause. A recent study of NHS2 participants by our research group observed a significant 17% lower risk of early menopause among women who consumed the highest vitamin D from food sources (Q5 median = 528 IU/d), as compared to those who consumed the least (Q1 median = 148 IU/d). (60) In contrast, we observed that total vitamin D intake was not associated with risk, whereas supplemental vitamin D intake (≥600 versus 0 IU/d) was associated with increased risk. The lack of strong and consistent associations of 25(OH)D with risk in the present analysis suggests that other dietary components or lifestyle factors correlated with vitamin D in foods may be associated with early menopause, rather than vitamin D itself. In our previous study, we noted a stronger association for dairy sources of vitamin D compared with non-dairy dietary vitamin D. Other constituents of dairy, such as lactose, calcium, or progesterone, which are highly correlated with vitamin D in dairy, may have influenced the observed inverse association for dietary vitamin D. The potential importance of dairy foods generally, rather than vitamin D specifically, is also supported by findings of Carwile et al, who observed that low-fat dairy intake was associated with later age at menopause, among women younger than 51 years of age. (8)

To answer this question, future studies of dairy foods and dairy constituents and risk of early menopause are warranted.

Vitamin D has been hypothesized to be related to ovarian aging and menopause timing primarily through effects on AMH and follicular development. Our null findings for 25(OH)D and AMH are consistent with those of three cross-sectional studies. (170, 171, 177) Pearce et al observed no association of 25(OH)D and AMH among 340 infertility treatment-seeking women <40 years of age living in South Australia. (170) Similarly, Drakopoulos et al also observed no association of 25(OH)D levels and AMH among an infertile population of 283 Belgian women <42 years of age, and Kim et al observed no association of 25(OH)D and AMH levels in 291 Korean women 35-49 years of age. (171, 177)

In contrast, two other studies reported significant associations between 25(OH)D and AMH. In a clinical trial among 33 premenopausal women living in New Zealand, Dennis et al observed seasonal variation of AMH levels mirroring that of 25(OH)D in both direction and magnitude, as well as a stabilizing effect of vitamin D_3 supplementation on the seasonality of AMH levels. (166) This is in contrast to placebo and vitamin D_2 supplementation groups, who experienced a statistically significant decline in AMH levels from summer to winter months. In another small trial, Naderi et al reported a statistically significant increase in both 25(OH)D levels and AMH levels after 3 months of vitamin D supplementation among 30 infertile Iranian women with vitamin D insufficiency or deficiency. (178) Likewise, in a cross-sectional study of US women, Mehri et al observed a statistically significant 1.1% higher log-transformed AMH for

each 1 ng/mL higher 25(OH)D level among 388 women at least 40 years of age, but no association among younger women. (115)

The inconsistency in findings between studies of 25(OH)D and AMH may potentially be explained by two important factors. First, it is possible that 25(OH)D may only be associated with changes in AMH levels among vitamin D deficient women. Both the Kim et al trial and the Merhi et al study were conducted among predominantly vitamin D deficient women. The Dennis et al trial did not provide baseline 25(OH)D levels of participants, but given the high latitude of residence of the study population, these women may have been vitamin D deficient prior to receiving the supplement or placebo, and thus supplementation may have been beneficial in preventing a seasonal decline in 25(OH)D and AMH levels. If a 25(OH)D-AMH association is only observable at the low end of the vitamin D spectrum, then one would expect to see an association only in populations with high prevalence of vitamin D deficiency, which the NHS2 is not. Given the small numbers of women with 25(OH)D levels below 35 nmol/L (n=28) in the NHS2, we were unable to address this possibility in our analyses.

It is also possible that the 25(OH)D-AMH relation may vary across racial groups due to differences in VDBP, as VDBP concentrations and binding affinity are determined almost entirely by genetic polymorphisms specific to race and ethnicity. (179) The binding affinity and concentration of VDBP is directly related to the bioavailability of 25(OH)D within the body, and thus may be biologically relevant to the mechanisms involved in ovarian aging. In our study, we observed a threshold of higher risk for early menopause among individuals in quartiles 2 through 4 of VDBP versus quartile 1, and a non-linear relation of VDBP with AMH levels, suggesting that VDBP levels and/or their

genetic determinants may be associated with risk. The NHS2, as well as the study populations of Pearce et al and Drakopolous et al. were almost entirely White, while that of Mehri et al was predominantly Black and Hispanic. If 25(OH)D is only associated with AMH in specific racial/ethnic groups, demographic differences in study populations, and thus the underlying population heterogeneity of VDBP polymorphisms, may provide some explanation for inconsistent findings.

Our ability to evaluate associations of early menopause and AMH with free 25(OH)D and VDBP in non-White individuals was limited for two reasons. First, given the racial homogeneity of the NHS2, we used a monoclonal rather than a polyclonal assay to measure VDBP, which has been shown to underestimate free 25(OH)D levels in non-White individuals. (180) Second, the NHS2 is comprised of mostly White women, and thus numbers of non-White individuals were too small to conduct race-stratified analyses. In light of evidence that the 25(OH)D-AMH relation may vary by race potentially due to VDBP differences, additional evaluation of 25(OH)D, VDBP and AMH in large, diverse populations is necessary.

It is important to note additional limitations of our study. First, we relied upon single measurements of 25(OH)D, VDBP, and AMH. Because 25(OH)D levels are influenced heavily by recent sun exposure and dietary intake, it is possible that within-person variability of 25(OH)D levels may have contributed measurement error, resulting in non-differential misclassification. However, in the NHS, a similar population of female health professionals, the intra-class correlation coefficients (ICC) for plasma 25(OH)D measured 2-3 years apart was 0.72 (P<0.001) (181), and for measurements 10 years apart was 0.51 (95% CI=0.42-0.60). (182) In a comparable population, the ICC for VDBP

measured over 1-3 years was 0.96 (P<0.001) (183), indicating that these biomarkers are relatively stable over time. Furthermore, while some researchers have raised questions about potential misclassification of vitamin deficiency status when 25OHD levels are measured by immunoassay, this technique is widely used and well validated for studies comparing disease risk across relative 25OHD levels, such as our study. (184) These factors help minimize the possibility that measurement error in biomarker data would explain our null findings for total and free 25(OH)D.

Second, we relied upon self-report of age at menopause, defined as the age at which periods were absent for 12 months, to ascertain cases and controls. Because women may experience amenorrhea and then have periods return again during perimenopause, some degree of measurement error in self-reported age at menopause is to be expected. Such measurement error would result in misclassification of early menopause cases and controls and produce a bias towards the null. However, among 6,591 women in the comparable NHS population, 82% of women reported the same age at menopause over multiple questionnaire cycles, suggesting high reproducibility. (168) Furthermore, we restricted the control group to women with age at menopause >48 years to reduce potential misclassification of the outcome. As such, misclassification of cases and controls would be an unlikely explanation for our findings.

Third, it is possible that residual confounding may have influenced our estimates. However, we were able to control for previously identified risk factors for early menopause such as smoking, vegetable protein intake, and BMI. None of the covariates included in multivariable models produced a >10% change in exposure estimates and

unadjusted estimates were similar to fully adjusted estimates, suggesting that substantial bias due to residual confounding is unlikely.

There are also several important strengths of our study to highlight. First, this was the first study to evaluate the association of 25(OH)D and early menopause. Not only did we assess total 25(OH)D, we also evaluated vitamin D binding protein and free 25(OH)D, which represents the biologically active vitamin D fraction and has been more strongly related to risk of some health outcomes than total 25(OH)D alone. Second, the size of our study population was larger than previous studies evaluating 25(OH)D and AMH, which provided higher statistical power and also allowed us to consider a wide variety of potential confounders. Finally, as most previous studies of 25(OH)D and AMH have been conducted among women seeking treatment for infertility, we anticipate that our findings are more widely generalizable to healthy premenopausal women. In the context of our findings and those of the aforementioned studies, it appears that 25(OH)D is unlikely to be related to AMH or early menopause, at least among White premenopausal women without vitamin D deficiency.

In conclusion, the findings of our study do not suggest that 25(OH)D levels are importantly related to risk of early menopause. Modest positive associations of VDBP levels with risk of early menopause and AMH levels warranted further evaluation in large, ethnically diverse populations.

Table 3.1 Characteristics of early menopause cases and controls at blood draw (1996-1999): Nurses' Health Study II

Characteristic[a]	Cases (n=328)	Controls (n=328)	*P*[b]
Age (y)[c]	40.2 (2.8)	40.2 (2.8)	0.99
BMI (kg/m^2)	25.4 (0.3)	25.0 (0.3)	0.10
Age at menarche (y)	12.4 (0.1)	12.3 (0.1)	0.57
Physical activity (MET-h/wk)	81.5 (14.7)	72.5 (12.9)	0.12
Parity	1.8 (0.1)	1.9 (0.1)	0.55
Duration of breastfeeding (mo)	4.8 (0.2)	5.0 (0.2)	0.25
Alcohol intake (g/d)	3.3 (0.3)	3.9 (0.4)	<0.01
Vegetable protein intake (% of total kcal/day)	5.2 (0.01)	5.5 (0.01)	0.09
Total vitamin D intake (IU/d)	348 (13.3)	381 (15.1)	0.03
Supplemental vitamin D intake	125 (10.9)	156 (11.7)	0.19
Dietary vitamin D intake	218 (6.5)	217 (6.4)	0.81
Dairy vitamin D intake	122 (6.2)	118 (5.7)	0.15
Total calcium intake (mg/d)	990 (24.5)	1062 (27.6)	0.03
Supplemental calcium intake	146 (15.7)	186 (19.7)	<0.001
Dietary calcium intake	841 (18.3)	865 (17.1)	0.29
Dairy calcium intake	537 (18.6)	542 (17.3)	0.27
Non-Hispanic White (%)	95.3	98.8	0.01
Season of blood draw (%)			0.64
Summer/fall	50.0	50.0	
Winter/spring	51.8	48.2	
Current smoker (%)	14.0	9.2	0.05
Smoking duration (pack-years)[d]	12.9 (1.0)	10.5 (0.7)	0.02
Current OC user (%)	1.8	3.7	0.15
OC use duration (mo)[e]	59.6 (3.2)	63.3 (3.5)	0.11

[a]Values are means (SE) unless otherwise indicated.
[b]P-values correspond to t-tests for continuous variables and chi-square tests for categorical variables.
[c]Values are means (SD).
[d]Among ever smokers only.
[e]Among ever OC users only.

Table 3.2 ORs (95% CIs) for early menopause according to quartile of total and free 25(OH)D and vitamin D binding protein levels: Nurses' Health Study II (1996-2011)

Biomarker	Quartile 1	Quartile 2	Quartile 3	Quartile 4	*P*-value[d]	Continuous[e]
Total 25(OH)D						
Median (nmol/L)	44.2	59.8	71.7	90.4		
Cases: Controls	86:83	92:83	71:82	79:80		
Unadjusted OR[a] (95% CI)	1	1.07 (0.70-1.64)	0.81 (0.51-1.29)	0.91 (0.56-1.48)	0.69	1.02 (0.83-1.25)
MV1[b] OR (95% CI)	1	1.21 (0.75-1.93)	0.93 (0.56-1.54)	1.04 (0.60-1.81)	0.75	1.12 (0.89-1.42)
MV2[c] OR (95% CI)	1	1.18 (0.73-1.89)	0.88 (0.53-1.46)	0.96 (0.55-1.69)	0.70	1.09 (0.85-1.38)
Free 25(OH)D						
Median (pmol/L)	13.9	18.7	24.2	32.4		
Cases: Controls	94:83	91:85	81:80	62:80		
Unadjusted OR[a] (95% CI)	1	0.92 (0.60-1.40)	0.87 (0.56-1.34)	0.65 (0.41-1.05)	0.32	0.82 (0.67-0.99)
MV1[b] OR (95% CI)	1	1.02 (0.64-1.62)	0.98 (0.61-1.57)	0.70 (0.41-1.20)	0.44	0.85 (0.68-1.05)
Vitamin D Binding Protein						
Median (μg/mL)	145.4	203.0	248.2	305.3		
Cases: Controls	56:81	87:81	86:83	99:83		
Unadjusted OR[a] (95% CI)	1	1.54 (0.99-2.41)	1.49 (0.96-2.34)	1.75 (1.11-2.77)	0.10	1.16 (0.99-1.36)
MV1[b] OR (95% CI)	1	1.54 (0.94-2.51)	1.80 (1.10-2.95)	1.80 (1.09-2.98)	0.07	1.17 (0.99-1.39)
MV2[c] OR (95% CI)	1	1.50 (0.92-2.46)	1.77 (1.08-2.90)	1.77 (1.06-2.94)	0.09	1.16 (0.98-1.38)

[a]Model adjusted for matching factors only (i.e., age at blood collection (within 4 mo), time of day of blood collection, month of collection (within 3 mo), sample type (luteal phase or untimed) and fasting status).

[b]Multivariable model 1 adjusted for matching factors + physical activity (<9, 9-<42, or ≥42 MET-h/wk), duration of breastfeeding at blood draw (0, >0-6, >6-18, >18 mo), smoking status at blood draw (non vs. current), BMI (<25 or ≥25 kg/m^2), and intakes of alcohol (0, 0.1-9.9, 10.0-29.9, or ≥30.0 g/day) and percent of vegetable protein (continuous) at blood draw.

[c]Multivariable model 2 adjusted for covariates in MV1 + mutual adjustment of total 25(OH)D and VDBP.

[d]P-values correspond to likelihood ratio tests comparing models with and without indicator variables for exposures.

[e]ORs correspond to 1 standard deviation increase in exposure.

Table 3.3 Unadjusted and adjusted AMH geometric means (95% CIs) according to quartile of total and free 25(OH)D and vitamin D binding protein: Nurses' Health Study II (1996-2011)

	N	Range	Unadjusted AMH Geometric Mean (95% CI) (ng/mL)	Adjusted[a] AMH Geometric Mean (95% CI) (ng/mL)
Total 25(OH)D		nmol/L		
Q1	169	22.9-52.1	0.4 (0.3-0.6)	0.5 (0.4-5.8)
Q2	175	52.2-65.8	0.4 (0.3-0.5)	0.4 (0.3-4.6)
Q3	153	65.9-78.8	0.4 (0.3-0.5)	0.4 (0.3-5.4)
Q4	159	78.8-159.0	0.4 (0.3-0.5)	0.4 (0.3-4.8)
P[b]			0.65	0.55
Free 25(OH)D		pmol/L		
Q1	177	6.8-16.2	0.4 (0.3-0.5)	0.4 (0.3-0.5)
Q2	176	16.3-21.4	0.4 (0.3-0.5)	0.4 (0.3-0.5)
Q3	161	21.6-27.5	0.4 (0.3-0.5)	0.4 (0.3-0.5)
Q4	142	27.5-88.9	0.5 (0.4-0.6)	0.5 (0.4-0.6)
P[b]			0.35	0.32
VDBP		µg/mL		
Q1	137	48.7-177.7	0.5 (0.4-0.7)	0.5 (0.4-0.7)
Q2	168	178.2-224.3	0.4 (0.3-0.5)	0.4 (0.3-0.6)
Q3	169	224.8-272.0	0.4 (0.3-0.5)	0.4 (0.3-0.5)
Q4	182	272.6-425.8	0.3 (0.3-0.4)	0.3 (0.3-0.4)
P[b]			0.07	0.04

[a]Multivariable model 1 adjusted for matching factors + physical activity (<9, 9-<42, or ≥42 MET-h/wk), duration of breastfeeding at blood draw (0, >0-6, >6-18, >18 mo), smoking status at blood draw (non vs. current), BMI (<25 or ≥25 kg/m^2), and intakes of alcohol (0, 0.1-9.9, 10.0-29.9, or ≥30.0 g/day) and percent of vegetable protein (continuous) at blood draw.
[c]P-value corresponds to type III *P*-value.

Table 3.4 ORs (95% CIs) for early menopause according to total 25(OH)D cutpoints: Nurses' Health Study II (1996-2011)

Total 25(OH)D	<50 nmol/L	50-74 nmol/L	≥75 nmol/L	*P*-value[e]
Median (nmol/L)	43.0	63.2	87.5	
Cases: Controls	68:70	164:158	96:100	
Unadjusted OR[a] (95% CI)	1	1.07 (0.71-1.59)	0.98 (0.61-1.56)	0.89
MV1[b] OR (95% CI)	1	1.29 (0.83-2.03)	1.15 (0.67-1.95)	0.50
MV2[c] OR (95% CI)	1	1.25 (0.79-1.96)	1.08 (0.63-1.85)	0.56

[a]Model adjusted for matching factors only (i.e., age at blood collection (within 4 mo), time of day of blood collection, month of collection (within 3 mo), sample type (luteal phase or untimed) and fasting status).

[b]Multivariable model 1 adjusted for matching factors + physical activity (<9, 9-<42, or ≥42 MET-h/wk), duration of breastfeeding at blood draw (0, >0-6, >6-18, >18 mo), smoking status at blood draw (non vs. current), BMI (<25 or ≥25 kg/m^2), and intakes of alcohol (0, 0.1-9.9, 10.0-29.9, or ≥30.0 g/day) and percent of vegetable protein (continuous) at blood draw.

[c]Multivariable model 2 adjusted for covariates in MV1 + mutual adjustment of total 25(OH)D and VDBP.

[e]P-values correspond to likelihood ratio tests comparing models with and without indicator variables for exposures.

CHAPTER 4

A PROSPECTIVE STUDY OF DAIRY FOOD INTAKE AND EARLY MENOPAUSE

4.1 Abstract

Early natural menopause, defined as the cessation of ovarian function prior to age 45, affects approximately 10% of women and increases risk of cardiovascular disease and other conditions. Laboratory evidence suggests a potential role of dairy foods in the ovarian aging process; however, no prior epidemiologic studies have evaluated how dairy intake is associated with risk of early menopause. We therefore evaluated how intakes of total, low-fat, high-fat and individual dairy foods are associated with early menopause in the Nurses' Health Study II. Women who were premenopausal at the start of follow-up in 1991 were followed until 2011 for early natural menopause. Food-frequency questionnaires were used to assess dietary intake. In Cox proportional hazards models adjusting for age, smoking, and other factors, total baseline dairy intake of ≥4 servings/day versus <4 servings/week was associated with 23% lower risk of early menopause (95% confidence interval (CI) = 0.64, 0.93; P-trend = 0.08). Associations appeared to be limited to low-fat dairy foods (≥2 servings/day versus <3 servings/month HR = 0.83; 95% CI = 0.68, 1.01; P-trend = 0.02), whereas high-fat dairy intake was not associated with early menopause. Low-fat dairy foods may represent a modifiable risk factor to reduce risk of early menopause among premenopausal women.

4.2 Introduction

Early natural menopause is defined as the cessation of ovarian function prior to age 45 and affects approximately 10% of women in Western populations. (1) Early menopause is associated with increased risk of several adverse health outcomes including cardiovascular disease, osteoporosis, depression, and early cognitive decline. (2–5) Furthermore, because female fertility declines substantially during the 10 years preceding the final menses, early menopause may also interfere with family planning. As women increasingly choose to delay childbearing until their later reproductive years, being unable to conceive as desired may have substantial psychological and financial implications. (1,6) Population-based studies indicate that genetic factors account for relatively little of the variation in menopausal timing and recent prospective studies have identified a number of modifiable risk factors for early menopause, including diet. (60,61,147) Bovine milk and dairy foods may be of particular interest, as they are comprised of a number of nutritive and non-nutritive components that may be physiologically related to ovarian aging and ovarian reserve. (unpublished manuscript; Purdue-Smithe et al., University of Massachusetts Amherst)

Milk is an excellent source of vitamin D and calcium, as well as other macro and micronutrients including dairy fat, dairy protein, lactose, vitamin A, B-vitamins, magnesium, phosphorus, potassium, and zinc. (65) Many of these nutrients, particularly vitamin D, have been hypothesized to be related to ovarian aging through potential effects on adiposity, inflammation, and anti-Müllerian hormone, a glycoprotein involved in follicle recruitment and a reliable proxy for ovarian reserve. (unpublished manuscript; Purdue-Smithe et al., University of Massachusetts Amherst) In addition, milk also

contains exogenous sex hormones including estrogens and progesterone. (66,87,185) The potential for dairy foods to influence levels of circulating hormones is supported by epidemiologic data suggesting that dairy consumption is positively associated with plasma levels of total and free estradiol. (86)

At this time, epidemiologic studies of milk and dairy consumption and menopause timing are scarce. We recently observed inverse associations of vitamin D and calcium intakes from food sources, particularly from dairy foods, and risk of early menopause in the Nurses' Health Study II (NHS2). (60) However, in a subsequent analysis, we observed no association of plasma 25-hydroxyvitamin D levels and risk of early menopause raising questions about whether associations observed with dietary and dairy vitamin D intake are instead explained by other components of dairy. (unpublished manuscript; Purdue-Smithe et al., University of Massachusetts Amherst) Despite these data, to our knowledge, no prior studies have specifically evaluated how intakes of dairy foods are associated with risk of early menopause (i.e., before 45). In light of this gap, the aim of the present study was to evaluate risk of early menopause with respect to intakes of total, low-fat, high-fat, and individual dairy foods among participants of the NHS2.

4.3 Subjects and Methods

The NHS2 is a prospective study of 116,429 female U.S. registered nurses who were 25-42 years old in 1989 when they responded to a mailed baseline questionnaire. Information regarding lifestyle behaviors and medical conditions are collected through biennial questionnaires, for which the follow-up rate for each cycle has been at least 89%.

The study protocol was approved by the Institutional Review Board at Brigham and Women's Hospital in Boston, MA.

4.3.1 Assessment of early menopause

On the 1989 baseline questionnaire, nurses were asked if their periods had ceased permanently with the following response options: 1) No: Premenopausal; 2) Yes: No menstrual periods; 3) Yes: had menopause but now have periods induced by hormones; and 4) Not sure; (e.g., started hormones prior to cessation of periods). Nurses who indicated that their periods had ceased were then asked the following questions: 1) At what age did your periods cease? (open response); and 2) For what reason did your periods cease? (response options were surgery; radiation or chemotherapy; and natural). Women were also asked about their current and past use of replacement sex hormones. Questions about menopausal status and replacement hormone therapy were repeated on all questionnaires thereafter. Age at natural menopause was defined as age after 12 consecutive months of amenorrhea not due to radiation, chemotherapy or surgery. A small number of women reported being postmenopausal on one questionnaire and then subsequently reported being premenopausal. For these women, we defined age at menopause as age after which periods were absent for 12 months or more, and then confirmed that this status persisted for at least 3 consecutive questionnaires.

We were interested in prospectively evaluating dairy intake and risk of early menopause; participants were thus eligible for inclusion in our study if they indicated being premenopausal and reported no age at menopause on the baseline 1989 questionnaire (n = 108,812). We then excluded women who did not respond to or who

reported implausible caloric intake (<500 or ≥3,500 kcal/d) on the 1991 food-frequency questionnaire (FFQ) (n = 22,847), were diagnosed with cancer before 1991 (n = 391), or whose date of menopause was before their return date of the 1991 FFQ (n = 283). After baseline exclusions, 85,651 women comprised the analytic study sample.

Women in the study sample were then followed prospectively until 2011 for self-report of the cessation of menses, as defined above, or first report of hysterectomy, bi-lateral or unilateral oophorectomy, cancer (not including non-melanoma skin cancer), loss to follow-up, or death. We identified cases of early menopause as women who reported natural menopause occurring before the age of 45.

4.3.2 Dietary assessment

Nurses were queried about their usual intake of 131 foods, beverages, and supplements over the preceding year via validated semi-quantitative FFQs in 1991, 1995, 1999, 2003, 2007, and 2011. (159–161) These FFQs asked participants to estimate, on average, how often they consumed specific foods and beverages. Participants reported their usual consumption by indicating one of nine frequency categories for each food and beverage (i.e., <1 serving/month, 1-3 servings/month, 1, 2-4, 5-6 servings/wk, and 1, 2-3, 4-5, and ≥6 servings/d). Specific dairy foods assessed by the FFQs included skim/low-fat milk, whole milk, cream, frozen yogurt/sherbet, yogurt, cottage/ricotta cheese, ice cream, cream cheese, other cheese, and butter. Total dairy intake was calculated by summing intakes of all dairy products. We calculated low-fat dairy intake by summing intakes of skim and non-fat milk, frozen yogurt/sherbet, yogurt, cottage/ricotta cheese, and other

low-fat cheese. High-fat dairy intake was equal to the sum of whole milk, cream, ice cream, cream cheese, other high-fat cheese, and butter intakes.

The validity of the FFQ was assessed by a comparison to 1-week diet records among a random subset (n = 173) of women in the Nurses' Health Study, a comparable population of female health professionals. For dairy foods, the Pearson correlation coefficients comparing the FFQ to a 1-week diet record were moderate to strong (range = 0.54-0.77), with the exception of hard cheese (r = 0.33). (160)

In 1998, 45,947 nurses completed a retrospective 124-item food frequency questionnaire that assessed their usual diet in high school. Dairy foods assessed on this questionnaire included chocolate milk, whole milk, low-fat milk, skim milk, yogurt, cottage or ricotta cheese, cheese, cream cheese, and butter. Total adolescent dairy intake was calculated as the sum of intakes of these foods. The reproducibility of this FFQ was assessed by a comparison to a subsequent FFQ assessing high school diet that was administered in 2002 to a subset of nurses who completed the initial FFQ (n=333). Reproducibility between the two questionnaires was high for dairy foods (r=0.64) and for milk specifically (r=0.76). (186)

4.3.3 Assessment of covariates

Information regarding age, race, height, ethnicity, maternal and paternal education level, physical activity during high school, body mass index at age 18, smoking during high school, and age at menarche was collected at baseline in 1989. Updated information on weight, parity, oral contraceptive use, breastfeeding, hormone therapy use, and smoking were collected biennially throughout follow-up. Baseline height and updated

weight were used to calculate updated body mass index (BMI) as weight (kg)/ height $(m)^2$ for each questionnaire cycle. Information on physical activity was collected in 1991, 1997, 2001, 2005, and 2009 using nurses' responses to questions regarding average time spent per week participating in specific activities (i.e., walking, running, biking, etc.), from which we calculated metabolic equivalent task (MET)-hours per week. (163)

Intakes of vegetable protein and alcohol were assessed via FFQ every four years beginning in 1991. Intakes of other nutrients present in dairy foods including dairy protein, lactose, dairy fat, dietary magnesium, phosphorus, potassium, zinc, vitamins B_1, B_2, B_5 and B_{12}, folate, calcium, vitamin D, and vitamin A were also estimated using these FFQs. For example, calcium intake from food sources was estimated by summing calcium content per 1 serving of each food and beverage (i.e., skim, low-fat, and whole milk, yogurt, hard cheese, cottage cheese, spinach, etc.) and multiplying it by the frequency of consumption. Intakes of vitamin D, vegetable protein, alcohol, and other nutrients were derived in a similar manner. We calculated percentage of total kcal from vegetable protein by multiplying grams of vegetable protein by 4 kcal/g and then dividing by total kcal.

Nurses were also asked to indicate their average use and dosage of multivitamins, calcium and vitamin D supplements every two years on FFQs or biennial questionnaires, which we used to estimate intakes of each nutrient from supplement sources. Total vitamin D and calcium intakes were then calculated by summing estimated dietary and supplemental intakes of each nutrient. We adjusted intakes of all nutrients for total energy using the residual method. (162)

4.3.4 Statistical analysis

We divided participants into categories of total dairy food intake and assessed baseline characteristics of our study sample according to category of total dairy food intake in 1991 using age-adjusted generalized linear models.

We then used Cox proportional hazards regression to estimate age-adjusted and multivariable hazard ratios (HR) for early menopause according to category of adult intake of total, high-fat, low-fat, and individual dairy foods. We repeated these analyses for adolescent intakes of total dairy and individual dairy foods. Tests for linear trend were conducted by modeling each exposure as a continuous servings/day variable. Participants contributed person-time (in months) beginning on the date of return of the 1991 questionnaire until menopause, first report of hysterectomy, bi-lateral or unilateral oophorectomy, cancer (not including non-melanoma skin cancer), loss to follow-up, or death, whichever occurred first. Analyses were stratified on age (in months) and questionnaire cycle.

We were interested in assessing potential variation in associations according to timing of exposure assessment and therefore modeled timing of intake using both baseline (1991) intake variables and cumulative average intake for each exposure. Cumulative average values for each exposure were calculated as mean intakes estimated from all FFQs up to and including the cycle prior to menopause.

There was very little evidence of confounding in our analyses, and thus covariate selection for multivariable models (MV1) was based on factors identified a priori (i.e., age, race/ethnicity, parity, multivitamin use, age at menarche, and physical activity) and factors previously identified as risk factors for early menopause in our population (i.e.,

smoking, BMI, duration of breastfeeding, and intakes of alcohol and vegetable protein). To assess whether estimates for dairy exposures may be confounded by intakes of vitamin D and calcium, which are associated with early menopause in NHS2, we additionally adjusted for intakes of total vitamin D and calcium in a second multivariable model (MV2). We also conducted analyses in which we further adjusted total dairy intake for intakes of dairy protein, lactose, dairy fat, dietary magnesium, phosphorus, potassium, zinc, vitamins B_1, B_2, B_5 and B_{12}, folate, and vitamin A in order to evaluate whether nutritive versus non-nutritive components of dairy may be more strongly associated with risk of early menopause. In multivariable models assessing adolescent dairy intake, we adjusted for factors identified a priori as potential confounders, including age, race/ethnicity, physical activity during high school, BMI at age 18, and smoking during high school.

We considered potential effect modification by BMI (underweight/normal vs. overweight/obese) and OC use (ever vs. never) on the total dairy-early menopause relation using likelihood ratio tests comparing models with and without multiplicative interactions terms.

We conducted a number of sensitivity analyses to evaluate the robustness of our estimates to potential residual confounding. Because some evidence suggests that polycystic ovary syndrome (PCOS) is related to both dairy intake and menopausal timing, we conducted analyses excluding women diagnosed with PCOS (n = 1,852). (187,188) We also conducted analyses restricted to women without diagnoses of autoimmune conditions including rheumatoid arthritis, multiple sclerosis, lupus, Crohn's disease, and ulcerative colitis, as these conditions are associated with earlier age at

menopause (189) and could be related changes in diet (n = 1,895). Furthermore, in order to assess the adequacy of our control for confounding by smoking, we restricted an analysis to never smokers (n = 1,226). Finally, we considered the possibility that medical conditions leading to hysterectomy may have also been related to dairy intake, and conducted analyses censoring at date of laparoscopy-confirmed endometriosis and ultrasound-confirmed uterine fibroid diagnosis in order to evaluate this potential selection bias.

All statistical analyses were conducted with SAS v9.4 software (SAS Institute Inc., Cary, NC). We used two-sided statistical tests performed at the 0.05 significance level for all analyses.

4.4 Results

Over 20 years of follow-up, 2,049 women in our study sample experienced early menopause. Age-adjusted baseline characteristics according to category of total dairy intake are presented in Table 4.1. At baseline, women reporting the highest dairy were on average younger, more physically active, less likely to smoke, and had higher BMI than those reporting the lowest intake. Dairy intake was also positively associated with, calcium, vitamin D and alcohol and inversely associated with vegetable protein intake.

In age-adjusted analyses, women who consumed ≥4 servings of total dairy per day versus <4 servings/week experienced a 26% (95% CI: 0.62-0.88) lower risk of early menopause (Table 4.2). In particular, women who consumed the most low-fat dairy foods (≥2 servings/day) were 24% (95% CI: 0.64-0.91) less likely to experience early menopause compared to women with the lowest intake (<3 servings/month). In contrast,

no association was observed for high-fat dairy intake (≥2 servings/day vs. <3 servings/month HR: 1.03; 95% CI: 0.87-1.23).

After adjusting for BMI, smoking, and other factors in multivariable model 1 (MV1), estimates for total and low-fat dairy intake were very similar, but slightly attenuated (Table 4.2). After further adjustment for total vitamin D and calcium intake in a second multivariable model (MV2), the HR comparing ≥4 per day versus <4 servings of total dairy per week was 0.77 (95% CI: 0.64-0.93). Each 1 serving/day higher intake of total dairy was associated with a marginally significant 3% (95% CI: 0.94-1.00; *P*-trend = 0.08) lower risk. Specifically, high (≥2 servings/day) versus low (<3 servings/month) intake of low-fat dairy foods was associated with 17% (95% CI: 0.68-1.01) lower risk of early menopause, and each 1 serving/day higher was associated with 5% (95% CI: 0.91-0.99; $P = 0.02$) lower risk. Similar to estimates from age-adjusted models, intake of high-fat dairy was not associated with risk of early menopause in multivariable analyses (<3 servings/month versus ≥2 servings/day MV2 HR: 1.03; 95% CI: 0.87-1.23). To evaluate potential confounding of low-fat dairy by high-fat dairy intake and vice versa, we also mutually adjusted these exposures in a third multivariable model (data not shown). Estimates from these models were substantively unchanged. Adjusting total dairy intake for intakes of dairy protein, lactose, dairy fat, dietary magnesium, phosphorus, potassium, zinc, vitamins B_1, B_2, B_5 and B_{12}, folate, and vitamin A did not meaningfully change the estimates for exposures (results not shown).

Our findings for total and low-fat dairy intake were consistent with those for low-fat dairy foods including skim milk and yogurt. For example, in fully adjusted models (MV2), each 1 serving per day of skim milk was associated with 6% (95% CI: 0.89-0.99;

P-trend = 0.02) lower risk of early menopause. Likewise, high (≥2 servings/day) versus low (almost never) intake of yogurt was associated with 14% (95% CI: 0.75-0.98) lower risk of early menopause (Table 4.4), although the *P*-trend was not significant (*P*-trend = 0.19). Other individual dairy foods were not significantly associated with risk of early menopause.

To evaluate how dairy intake over longer periods of time may be associated with early menopause, we also conducted analyses in which we modeled cumulative average intake of exposures. Estimates from these models were similar to those of baseline (1991) exposures, but attenuated slightly. For example, in models evaluating cumulative average intake, each 1 serving/day increase in total dairy and low-fat dairy intake was associated with 2% (95% CI: 0.94-1.01; P-trend = 0.15) and 5% (95% CI: 0.90-1.00; P-trend = 0.04) lower risk of early menopause, respectively (complete data not shown).

Our findings for adolescent intake of dairy foods and risk of early menopause among the subset (n=1,012) of women who completed high school diet questionnaires are presented in Table 4.3. Dairy intake was substantially higher during adolescence (median = 10 servings/d) than adulthood (median = 2 servings/d) and therefore ranges were not comparable between time periods. Although analyses of dairy intake during adolescence were ultimately underpowered, estimates were similar to those for adult dairy intakes. For example, in multivariable analyses adjusting for BMI at age 18, smoking in high school, and other factors, the HR comparing total adolescent dairy intake of ≥6 versus <4 servings/d was 0.86 (95% CI: 0.66-1.12). We also conducted analyses including total dairy intake during adolescence and at baseline (1991) in the same model to evaluate whether adolescent dairy intake may be differently related to early menopause risk than

adult dairy intake. Estimates for exposures at both time periods were similar but slightly stronger after mutual adjustment.

Analyses restricted to women without PCOS or autoimmune conditions and women who never smoked produced estimates similar to those among the full population. There was no evidence of multiplicative interaction by OC use (P-interaction = 0.53) or BMI (P-interaction = 0.68) on the total dairy-early menopause relation.

4.5 Discussion

In this prospective study, we found intake of low-fat dairy foods to be inversely associated with risk of early menopause. The observed inverse association of low-fat dairy intake appears to be strongly related to intakes of skim milk and yogurt, as these foods, but not others, were associated with lower risk of early menopause. We also observed some suggestion of a possible inverse association of risk with low-fat dairy intake in adolescence, though lower sample size for these analyses limited our statistical power. In contrast, high-fat dairy intake at either time point did not appear related to risk.

To our knowledge, this is the first study to evaluate how dairy consumption is specifically related to risk of early menopause. However, our findings are largely consistent with those of a recent prospective study evaluating dairy and overall menopause timing. In this study, Carwile et al observed that low-fat dairy and skim milk intakes, but not total or high-fat dairy intake, were associated with earlier age at menopause only among participants of the NHS who were <51 years of age. (8) Conversely, Nagel et al observed no association of total dairy intake and age at menopause among 5,110 participants of the EPIC cohort. (131) The inconsistency of our

findings for total dairy intake versus those of Carwile and Nagel is notable, but may be explained by overall higher intakes of high-fat dairy in these populations.

A number of different mechanisms relating constituents of dairy to ovarian aging have been proposed, including upregulation of anti-Müllerian hormone by vitamin D and potential effects on vitamin D-mediated inflammatory pathways. (unpublished manuscript; Purdue-Smithe et al., University of Massachusetts Amherst) In our recent analyses, we observed that vitamin D and calcium intakes from food sources, specifically from dairy foods, were associated with lower risk of early menopause among participants of the NHS2. (60) We subsequently found that plasma 25(OH)D levels were not associated with risk of early menopause or AMH levels in our population, suggesting that our findings are more likely to be explained by mechanisms involving other components of milk, rather than those involving vitamin D. (unpublished manuscript; Purdue-Smithe et al., University of Massachusetts Amherst)

Alternatively, the observed associations may be explained by alterations in sex hormone levels by non-nutritive components of dairy. Milk products contain varying concentrations of conjugated and unconjugated estrogen metabolites and progesterone. (66,185) Higher concentrations of lipophilic unconjugated estrogens and progesterone are present in high-fat dairy products, whereas hydrophilic conjugated estrogens are more concentrated in low-fat dairy products. (86,185) Hydrophilic conjugated estrogen metabolites, such as estrone sulfate, are considered to be more biologically active than their unconjugated counterparts due to their circumvention of hepatic metabolism. (84) Differences in our findings for low-fat and high-fat dairy intake may thus be explained by

the relative bioavailability and concentration of these hormones depending upon milk fat content.

Milk also contains androgens such as testosterone and androstenedione, which may be implicated in ovarian aging. (87) Epidemiologic evidence suggests that exogenous androgens are positively associated with circulating insulin-like growth factor 1 (IGF-1) in humans. (88) Age is associated with a decrease in circulating IGF-1, and studies in rats have observed that low IGF-1 is associated with disruption of luteinizing hormone, which regulates ovulation. (8,93) Dairy consumption may therefore increase levels of IGF-1, potentially allowing for the continuation of normal menstrual cycles during the later reproductive years.

The potential for dairy food intake to influence levels of hormones in humans is evidenced by epidemiologic studies showing that consumption of dairy products is positively associated with plasma levels of total and free estradiol and IGF-1. (86,89,90,92) Indeed, milk intake has been associated with other reproductive outcomes including endometriosis (13) and premenstrual syndrome (63), as well as acne (190), suggesting that the levels of hormones present in dairy are sufficient to alter circulating levels and in turn, possibly influence risk of health outcomes. Given that associations for low-fat dairy intake persisted after we controlled for vitamin D and calcium intakes, as well as other nutrients in milk, the mechanisms involving hormones in milk seem to be the most likely physiologic explanation for our findings.

Strengths of our study include large sample size, prospective design, and high retention of participants throughout the duration of follow-up (>89%). Our study also has several limitations to note. First, we used nurses' self-report of age at menopause to

classify cases and non-cases. Some women may have misreported their age at menopause, resulting in misclassification of case status. However, self-reported menopausal status has been shown to be a reproducible method of assessment over multiple questionnaire cycles in a comparable population of female nurses. (168) Among 6,591 NHS women who were premenopausal in 1976 and reported having natural menopause on the 1978 questionnaire, 82% reported their age at menopause to within 1 year on the following two questionnaires, suggesting that our findings are likely robust to substantial misclassification of outcome. Importantly, any misclassification of case status is unlikely to be related to dairy intake and would not explain our positive findings. Second, some degree of non-differential misclassification due to error in self-reported dairy food intake is expected. However, misclassification across extreme categories of dairy intake is improbable and would likely produce a bias towards the null.

Finally, estimates for exposures assessed at baseline were stronger than those in cumulative average models, perhaps suggesting that dairy intake may be more strongly related to early menopause risk at younger ages. However, we were unable to assess valid age-specific estimates for updated adult dairy intake and early menopause due to models that used age as the underlying time variable to evaluate an age-dependent outcome.

There are also limitations specific to our analyses of adolescent intakes of dairy. Although estimates from models evaluating adolescent intake of total dairy are similar to those of adult intakes, our ability to make direct comparisons across time periods is limited due to lower sample size for adolescent and childhood analyses and substantially higher overall total dairy intake levels. Also, intake levels of dairy during adolescence and adulthood were highly correlated in our population, making it difficult to

differentiate temporally whether observed associations for each time point are independent. Future large prospective studies may be more adequately powered to evaluate how early life dairy intake may be associated with risk of early menopause.

The NHS2 is a heterogeneous population with regard to many lifestyle and dietary variables; we therefore anticipate that our findings would apply to similar groups of premenopausal women. However, our findings may not be applicable to women who cannot consume dairy products due to milk allergies or lactose-intolerance. Findings of our study indicate that low-fat dairy products including skim milk and yogurt may represent modifiable risk factors for women to reduce risk of early menopause.

Table 4.1 Age-adjusted characteristics of premenopausal women according to category of total dairy intake at baseline (1991): Nurses' Health Study II, 1991[a]

	Total Dairy Intake (servings)					
Characteristic	<2/wk (n=2091)	2-4/wk (n=5334)	5-6/wk (n=6062)	1/d (n=28419)	2-3/d (n=32429)	≥4/d (n=11899)
Age, y [b]	36.8 (4.5)	36.5 (4.6)	36.3 (4.6)	36.0 (4.6)	35.6 (4.6)	35.2 (4.5)
BMI, kg/m^2	24.1 (0.1)	24.5 (0.1)	24.5 (0.01)	24.5 (0.03)	24.5 (0.03)	24.6 (0.05)
Calcium intake, mg/d	711 (8.5)	728 (5.3)	785 (5.0)	894 (2.3)	1127 (2.2)	1304 (3.6)
Vitamin D intake, IU/d	302 (5.6)	295 (3.5)	318 (3.3)	349 (1.5)	428 (1.4)	476 (2.3)
Age at menarche, y	12.4 (0.03)	12.4 (0.02)	12.4 (0.02)	12.4 (0.01)	12.4 (0.01)	12.5 (0.01)
Full-term pregnancies, *n*	1.4 (0.03)	1.4 (0.02)	1.5 (0.02)	1.5 (0.01)	1.6 (0.01)	1.7 (0.01)
Physical activity, MET-h/wk	22.3 (1.4)	21.4 (0.9)	22.7 (0.8)	23.7 (0.4)	24.7 (0.4)	26.7 (0.6)
Vegetable protein intake, % of total kcal	5.6 (0.02)	5.3 (0.01)	5.2 (0.01)	5.1 (0.01)	4.9 (0.01)	4.6 (0.01)
Alcohol intake, g/d	2.6 (0.1)	3.0 (0.1)	3.2 (0.1)	3.2 (0.04)	3.1 (0.03)	3.3 (0.1)
Ever used OCs, %	82	85	85	85	84	82
Current smoker, %	17	15	14	12	10	12

[a]Values are means ± SEs or percentages, unless otherwise indicated. All characteristics were calculated with the use of generalized linear models adjusted for the age of participants in 1991. MET-h, metabolic equivalent task hours; Q, quintile.
[b]Values are means ± SDs.

Table 4.2 HRs (95% CIs) for early menopause by category of baseline (1991) intake of total, high-fat and low-fat dairy, and individual dairy foods: Nurses' Health Study II (1991-2011)

	Cases	Age-adjusted HR (95% CI)	MV1[a] HR (95% CI)	MV2[b] HR (95% CI)
Total dairy				
≤4/wk	214	1	1	1
5-6/wk	160	0.92 (0.75-1.13)	0.94 (0.77-1.16)	0.94 (0.76-1.15)
1/d	673	0.81 (0.69-0.94)	0.84 (0.72-0.98)	0.83 (0.71-0.98)
2-3/d	736	0.75 (0.65-0.88)	0.81 (0.70-0.95)	0.80 (0.68-0.93)
≥4/d	266	0.74 (0.62-0.88)	0.79 (0.66-0.95)	0.77 (0.64-0.93)
per 1 serving/d		0.96 (0.94-0.99)	0.98 (0.95-1.01)	0.97 (0.94-1.00)
P		0.01	0.1	0.08
High-fat dairy				
<3/m	297	1	1	1
1/wk	451	1.00 (0.86-1.15)	1.03 (0.89-1.19)	1.03 (0.89-1.19)
2-4/wk	441	0.92 (0.79-1.07)	0.96 (0.83-1.12)	0.96 (0.83-1.12)
5-6/wk	212	0.93 (0.78-1.11)	0.98 (0.82-1.17)	0.98 (0.82-1.17)
1/d	413	0.96 (0.83-1.11)	1.00 (0.86-1.17)	1.00 (0.86-1.17)
≥2/d	235	1.03 (0.87-1.23)	1.03 (0.87-1.22)	1.03 (0.87-1.23)
per 1 serving/d		1.01 (0.97-1.06)	1.00 (0.96-1.04)	1.00 (0.96-1.05)
P		0.51	0.85	0.83
Low-fat dairy				
<3/m	158	1	1	1
1/wk	242	0.97 (0.79-1.19)	1.01 (0.83-1.24)	1.01 (0.83-1.24)
2-4/wk	316	0.92 (0.76-1.12)	0.99 (0.81-1.20)	0.98 (0.81-1.19)
5-6/wk	167	0.81 (0.65-1.01)	0.87 (0.70-1.09)	0.86 (0.69-1.08)
1/d	618	0.80 (0.67-0.95)	0.88 (0.74-1.05)	0.86 (0.72-1.03)
≥2/d	548	0.76 (0.64-0.91)	0.87 (0.72-1.04)	0.83 (0.68-1.01)
per 1 serving/d		0.93 (0.90-0.97)	0.96 (0.93-1.00)	0.95 (0.91-0.99)
P		<0.01	0.05	0.02
Low-fat dairy foods				
Skim milk				
per 1 serving/d		0.92 (0.88-0.97)	0.96 0.91-1.00)	0.94 (0.89-0.99)
P		<0.01	0.04	0.02
Yogurt				
per 1 serving/d		0.86 (0.71-1.04)	0.88 (0.73-1.07)	0.88 (0.72-1.07)
P		0.13	0.19	0.19
Frozen yogurt/sherbet				
per 1 serving/d		0.91 (0.74-1.11)	0.95 (0.78-1.16)	0.95 (0.78-1.16)
P		0.35	0.61	0.61
Cottage/ricotta cheese				
per 1 serving/d		1.01 (0.76-1.36)	1.08 (0.81-1.43)	1.08 (0.81-1.43)
P		0.92	0.61	0.61

Low-fat other cheese			
per 1 serving/d	1.00 (0.84-1.20)	1.03 (0.86-1.22)	1.03 (0.86-1.23)
P	0.97	0.79	0.78
High-fat dairy foods			
Whole milk			
per 1 serving/d	1.09 (0.96-1.23)	1.05 (0.92-1.19)	1.05 (0.92-1.19)
P	0.19	0.46	0.46
Cream			
per 1 serving/d	1.04 (0.97-1.11)	1.00 (0.94-1.07)	1.00 (0.94-1.07)
P	0.26	0.97	0.96
Ice cream			
per 1 serving/d	0.81 (0.62-1.04)	0.91 (0.72-1.17)	0.92 (0.72-1.17)
P	0.09	0.47	0.47
Cream cheese			
per 1 serving/d	0.83 (0.59-1.18)	0.88 (0.63-1.24)	0.88 (0.63-1.24)
P	0.31	0.47	0.48
High-fat other cheese			
per 1 serving/d	1.00 (0.90-1.11)	1.04 (0.94-1.16)	1.04 (0.94-1.16)
P	0.99	0.46	0.44
Butter			
per 1 serving/d	1.01 (0.91-1.11)	0.99 (0.89-1.09)	0.99 (0.89-1.08)
P	0.92	0.78	0.78

[a]MV1 model adjusted for age (months; continuous), pack-years of smoking (0-10, 11-20, or ≥21), BMI (in kg/m^2; <18.5, 18.5 to <25, 25 to <30, or ≥30), age at menarche (continuous), parity (nulliparous, 1-2, or ≥3), breastfeeding duration (continuous), percentage of total kcal from vegetable protein (quintiles 1-3 or 4+5), alcohol intake (<10 or ≥10 g/d), and current multivitamin use (yes or no).

[b]MV2 model adjusted for MV1 covariates + total vitamin D intake (continuous) and total calcium intake (continuous).

Table 4.3 HRs (95% CIs) for early menopause by category of adolescent intake of total and individual dairy foods: Nurses' Health Study II (1991-2011)

	Cases	Age-adjusted HR (95% CI)	MV1[b] HR (95% CI)
Total dairy			
<4/d	60	1	1
4-5/d	112	0.92 (0.67-1.27)	0.93 (0.68-1.28)
6+/d	840	0.86 (0.66-1.11)	0.86 (0.66-1.12)
per 1 serving/d		1.00 (0.99-1.01)	1.00 (0.99-1.01)
P		0.60	0.64
Skim milk			
Almost never	748	1	1
1/m-1/d	135	0.90 (0.74-1.08)	0.92 (0.76-1.11)
2+/d	129	0.89 (0.74-1.08)	0.92 (0.76-1.11)
per 1 serving/d		0.95 (0.89-1.02)	0.96 (0.90-1.03)
P		0.17	0.29
Whole milk			
Almost never	414	1	1
1/m-6/wk	207	1.10 (0.92-1.30)	1.07 (0.90-1.27)
1/d	118	0.99 (0.81-1.22)	0.98 (0.80-1.20)
2+/d	273	1.02 (0.87-1.19)	1.01 (0.86-1.18)
per 1 serving/d		1.00 (0.95-1.05)	1.00 (0.95-1.05)
P		0.95	0.89

[b]Multivariable model 1 adjusted for age (continuous months), race (white or non-white), physical activity during high school (continuous MET-h/wk), BMI at age 18 (continuous kg/m^2), and smoking during high school (continuous pack-years).

Table 4.4 HRs (95% CIs) for early menopause by category of baseline (1991) intake of individual dairy foods: Nurses' Health Study II (1991-2011)

	Cases	Age-adjusted HR (95% CI)	MV1[a] HR (95% CI)	MV2[b] HR (95% CI)
Skim milk				
Almost never	324	1	1	1
1-3/m	168	0.97 (0.80-1.17)	1.00 (0.83-1.20)	1.00 (0.82-1.20)
1/wk	161	1.08 (0.89-1.30)	1.12 (0.92-1.35)	1.11 (0.92-1.35)
2-4/wk	322	0.81 (0.69-0.94)	0.85 (0.73-0.99)	0.85 (0.72-0.99)
5-6/wk	161	0.81 (0.67-0.98)	0.85 (0.71-1.03)	0.84 (0.69-1.02)
1/d	471	0.83 (0.72-0.95)	0.89 (0.77-1.03)	0.88 (0.76-1.01)
2-3/d	399	0.74 (0.64-0.86)	0.83 (0.71-0.96)	0.79 (0.67-0.93)
≥4/d	43	0.91 (0.66-1.25)	1.03 (0.75-1.42)	0.96 (0.69-1.35)
P		<0.01	0.04	0.02
Whole milk				
Almost never	1656	1	1	1
1-3/m	171	1.05 (0.90-1.23)	1.02 (0.87-1.20)	1.02 (0.87-1.20)
1-4/wk	46	1.08 (0.91-1.28)	1.03 (0.87-1.22)	1.03 (0.87-1.22)
≥5/wk	76	1.05 (0.83-1.32)	0.98 (0.78-1.23)	0.98 (0.78-1.23)
P		0.19	0.46	0.46
Cream				
Almost never	1441	1	1	1
1-3/m	297	0.95 (0.84-1.08)	0.97 (0.85-1.10)	0.97 (0.85-1.10)
1/wk	67	0.99 (0.77-1.27)	1.01 (0.79-1.29)	1.01 (0.79-1.29)
2-4/wk	70	1.06 (0.84-1.35)	1.06 (0.84-1.35)	1.06 (0.84-1.35)
≥5/wk	174	1.05 (0.90-1.23)	0.99 (0.85-1.16)	0.99 (0.85-1.16)
P		0.26	0.97	0.96
Frozen yogurt/sherbet				
Almost never	682	1	1	1
1-3/m	739	0.96 (0.86-1.06)	1.00 (0.90-1.11)	1.00 (0.90-1.11)
1/wk	315	0.94 (0.82-1.07)	1.00 (0.87-1.14)	1.00 (0.87-1.14)
≥2/wk	313	0.94 (0.82-1.07)	0.99 (0.87-1.13)	0.99 (0.87-1.14)
P		0.35	0.61	0.61
Ice cream				
Almost never	611	1	1	1
1-3/m	862	1.01 (0.91-1.11)	1.06 (0.95-1.18)	1.06 (0.95-1.18)
1/wk	346	0.99 (0.87-1.13)	1.08 (0.94-1.23)	1.08 (0.94-1.23)
≥2/wk	230	0.89 (0.76-1.03)	0.98 (0.84-1.15)	0.98 (0.84-1.15)
P		0.09	0.47	0.47
Yogurt				
Almost never	956	1	1	1
1-3/m	538	0.86 (0.77-0.95)	0.90 (0.81-1.00)	0.89 (0.80-1.00)
1/wk	251	0.94 (0.81-1.07)	0.99 (0.86-1.14)	0.99 (0.86-1.13)
≥2/wk	304	0.83 (0.73-0.95)	0.86 (0.75-0.98)	0.86 (0.75-0.98)

P		0.13	0.19	0.19
Cottage/ricotta cheese				
Almost never	933	1	1	1
1-3/m	742	0.94 (0.86-1.04)	0.98 (0.89-1.08)	0.98 (0.89-1.08)
1/wk	216	0.94 (0.81-1.09)	0.99 (0.85-1.15)	0.99 (0.85-1.15)
≥2/wk	158	1.04 (0.88-1.23)	1.08 (0.91-1.28)	1.08 (0.91-1.28)
P		0.92	0.61	0.61
Cream cheese				
Almost never	1109	1	1	1
1-3/m	654	0.93 (0.84-1.02)	0.96 (0.87-1.05)	0.96 (0.87-1.05)
1/wk	172	0.89 (0.76-1.05)	0.94 (0.80-1.10)	0.94 (0.80-1.10)
≥2/wk	114	0.94 (0.78-1.14)	0.97 (0.80-1.18)	0.97 (0.80-1.18)
P		0.31	0.47	0.48
Low-fat cheese				
Almost never	1551	1	1	1
1-3/m	85	1.08 (0.86-1.36)	1.06 (0.84-1.34)	1.06 (0.84-1.34)
1/wk	111	1.03 (0.85-1.26)	1.03 (0.84-1.25)	1.03 (0.84-1.25)
≥2/wk	214	0.98 (0.86-1.12)	1.00 (0.87-1.15)	1.00 (0.87-1.15)
P		0.97	0.79	0.78
High-fat cheese				
Almost never	721	1	1	1
1-3/m	169	0.94 (0.79-1.12)	0.93 (0.77-1.11)	0.93 (0.77-1.11)
1/wk	274	1.05 (0.92-1.21)	1.07 (0.93-1.23)	1.07 (0.93-1.23)
≥2/wk	714	1.02 (0.92-1.12)	1.07 (0.97-1.19)	1.07 (0.97-1.19)
P		0.99	0.46	0.44
Butter				
Almost never	1284	1	1	1
1-3/m	420	1.02 (0.91-1.14)	1.00 (0.89-1.11)	1.00 (0.89-1.11)
≥1/wk	345	1.01 (0.89-1.14)	0.97 (0.86-1.09)	0.97 (0.86-1.09)
P		0.92	0.78	0.78

[a]MV1 model adjusted for age (months; continuous), pack-years of smoking (0-10, 11-20, or ≥21), BMI (in kg/m^2; <18.5, 18.5 to <25, 25 to <30, or ≥30), age at menarche (continuous), parity (nulliparous, 1-2, or ≥3), breastfeeding duration (continuous), percentage of total kcal from vegetable protein (quintiles 1-3 or 4+5), alcohol intake (<10 or ≥10 g/d), and current multivitamin use (yes or no).

[b]MV2 model adjusted for MV1 covariates + total vitamin D intake (continuous) and total calcium intake (continuous).

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